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THE EFFECTS OF AN AMINO ACID MIXTURE BEVERAGE ON GLUCOSE TOLERANCE, GLYCOGEN REPLENISHMENT, MUSCLE DAMAGE, AND ANAEROBIC EXERCISE PERFORMANCE

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THE EFFECTS OF AN AMINO ACID MIXTURE BEVERAGE ON GLUCOSE TOLERANCE, GLYCOGEN REPLENISHMENT, MUSCLE DAMGE, AND ANAEROBIC EXERCISE PERFORMANCE

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Recent research suggests that amino acids, such as leucine and isoleucine, can improve glucose tolerance in vivo and in vitro animal models by accelerating glucose uptake in peripheral tissues and stimulate glycogen synthesis in vitro in the absence of insulin. Our laboratory recently found that gavaging normal Sprague-Dawley rats with an amino acid mixture, composed of isoleucine, leucine, cystine, methionine, and valine, improved blood glucose response during an oral glucose challenge without an increase in the plasma insulin response. The blood glucose-lowering effect of the amino acid mixture was due to an increase in skeletal muscle glucose uptake. These results suggest that this amino acid supplement acutely improves muscle insulin sensitivity and blood glucose homeostasis. However, the effect of this amino acid mixture on glucose tolerance and muscle glycogen synthesis in humans has not been investigated. Some studies have also shown that daily supplementation or acute ingestion of amino acids may prevent muscle damage that occurs as a result of a prolonged, intense endurance exercise or strength training and therefore improves force production and exercise performance. However, the effects of the addition of an amino acid mixture to carbohydrate supplement on muscle damage after a prolonged endurance exercise, as well as on the subsequent anaerobic

exercise performance, have not been characterized. Therefore, in this series of two studies, the effects of an amino acid mixture, composed of isoleucine, leucine, cyctine, methionine, and valine, on glucose tolerance, muscle glycogen resynthesis, muscle damage, and anaerobic exercise performance were investigated. Study 1 demonstrated that our amino acid mixture lowered the glucose response to an OGTT in healthy overweight/obese subjects in an insulin-independent manner. Study 2 demonstrated that both high and low dosages of amino acid mixture were effective in lowering blood glucose response to a carbohydrate bolus in athletes postexercise. High dosage of amino acid mixture was more potent in glucose regulation by providing a higher insulin response and amino acid effect. However, our amino acid mixture had no effects on post exercise muscle glycogen synthesis, exercise-induced muscle damage or subsequent anaerobic performance. Taken together, the results of this research series suggest that an amino acid mixture, composed of isoleucine and 4 additional amino acids, attenuates the glucose response to a glucose bolus in an insulin-independent manner, but does not enhance muscle glycogen restoration following exercise or prevent exercise-induced muscle damage.

Table of Contents

List of Tables

List of Figures

Chapter I: General Introduction

Amino acids are the building blocks of proteins, and as such they are essential for the synthesis of structural proteins, enzymes, some hormones and neurotransmitters. Moreover, amino acids are also involved in numerous metabolic pathways. One of these metabolic pathways mediated by amino acids is for glucose transport. Recent *in vivo* and *in vitro* animal studies suggest that amino acids, such as leucine and isoleucine, may lower the blood glucose level by accelerating glucose uptake in skeletal muscle, which is insulin-independent (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007; Kalogeropoulou et al., 2008; Morifuji et al., 2009; Nishitani et al., 2002). The hypoglycemic effect of isoleucine holds great clinical significance for individuals with impaired glucose tolerance. The ability of isoleucine to lower blood glucose independently of insulin may provide a non-pharmaceutical therapy in the fight against insulin resistance and type 2 diabetes mellitus.

One possible factor that may have contributed to the potential hypoglycemic effect of amino acids is an acceleration on peripheral tissue glucose uptake. It is well-known that skeletal muscle is the predominate tissue for the clearance of blood glucose and 70 to 80% of an oral glucose challenge is cleared by skeletal muscle (DeFronzo et al., 1981; Katz et al., 1983; Willems et al., 1991). The storage form of glucose in the muscles, glycogen, is an important fuel source for moderate to high intensity exercise. It has been clearly demonstrated that aerobic endurance is directly related to the initial muscle glycogen stores (Bergstrom et al., 1967; Bergstrom and Hultman, 1966) and that strenuous exercise cannot be maintained once these stores are depleted (Bergstrom et al., 1967; Bergstrom and Hultman, 1966; Hermansen et al., 1967). During intense physical training and tournament competitions, it is very important to speed up the recovery process when recovery time is limited and achieve optimal performance in up coming exercise. The faster the muscle glycogen can be replenished post-exercise, the greater ability to achieve optimal performance in the subsequent exercise. Therefore, amino acid supplementation during the recovery period may be beneficial for rapid and effective

replenishment of muscle glycogen and subsequent exercise performance, taking account of the hypoglycemic effects of amino acids.

Another important aspect of endurance exercise recovery is muscle damage repair. Muscle damage not only limits performance due to delayed onset muscle soreness (DOMS), but it also compromises the muscle glycogen replenishment and limits muscle training adaptations (Costill et al., 1990; O'Reilly et al., 1987). This damage does not just occur during exercise, but can continue after exercise for many hours. It is well known that immediately after prolonged, intense endurance exercise, the body is in a catabolic physiological state in which blood insulin is low and cortisol and the catecholamines are high. Although there is an increase in muscle protein synthesis, its rate is exceeded by the rate of protein degradation such that there is a negative net protein balance. During the catabolic physiological state, considerate muscle tissue damage can occur. Proper nutrient supplementation post-exercise can shift a catabolic physiological state to an anabolic state. The ingestion of carbohydrate and protein or amino acids following exercise has been reported to promote protein synthesis and hasten recovery post-exercise, taking advantage of an enhanced insulin response, energy availability, and substrate for protein synthesis (Biolo et al., 1997; Koopman et al., 2005; Levenhagen et al., 2001; Tipton et al., 1999). Based on these studies in which an anabolic response was promoted by carbohydrate-protein/amino acids supplementation, it could be predicted that such supplementation would also reduce exercise-induced muscle damage.

During intense physical training and tournament competitions, rapid recovery from the previous exercise is essential for achieving optimal performance in the subsequent exercise bout. Muscle damage that occurs as a result of a prolonged, intense endurance exercise could affect force production and result in a reduction in the subsequent anaerobic exercise performance. Some studies also suggest that muscle glycogen status influences anaerobic power generation and capacity for high-intensity exercise (Lacombe et al., 2001; Langfort et al., 1997). Therefore, anaerobic performance during a subsequent exercise bout maybe highly influenced by nutrient supplementation.

Therefore, this series of studies aimed to investigate the effects of an amino acid mixture on glucose metabolism and exercise recovery in humans. Study 1 investigated whether the amino acid mixture attenuates blood glucose response during a glucose

challenge in healthy overweight/obese adults. An overweight/obese population was chosen because weight is considered the single strongest predictor of type 2 diabetes. After we found a significant effect of the amino acid mixture on blood glucose regulation, we continued to investigate the effects of the same amino acid mixture on blood glucose homeostasis, muscle glycogen synthesis, muscle damage markers, and subsequent anaerobic performance post prolonged exercise in trained athletes. In addition, we examined whether this amino acid mixture can better activate cellular signaling proteins that regulate glucose transport and glycogen synthesis. Because it has been reported that amino acids cause gastrointestinal (GI) distress in some individuals, we compared the effects of half dosage with full dosage of amino acid mixture as used in Study 1 and investigated whether half dosage had the same effect on glucose tolerance but without any GI distress.

OBJECTIVES

Study 1: to determine whether the ingestion of an amino acid mixture, composed of isoleucine and 4 additional amino acids, could improve the blood glucose response of healthy overweight/obese men and women following an oral glucose tolerance test.

Study 2: to determine whether an amino acid mixture, composed of isoleucine and 4 additional amino acids, ingested with a carbohydrate beverage, could improve the blood glucose response of healthy active men and women postexercise. We also investigated whether the rate of muscle glycogen synthesis following strenuous exercise is increased with this amino acid+carbohydrate beverage (CHO/AA) given post-exercise, as compared to a carbohydrate only beverage (CHO). Furthermore, we investigated the activation states of signaling proteins that control both muscle glucose uptake and glycogen synthesis, the effect of the CHO/AA supplement on exercise-induced muscle damage, and subsequent anaerobic exercise performance. Two different doses of the amino acid mixture in combination with carbohydrate supplementation (CHO/HAA, CHO/LAA) were tested.

HYPOTHESES

Study 1

1. In comparison with a placebo, an amino acid mixture, composed of isoleucine and 4 additional amino acids, will significantly reduce the blood glucose response to a glucose challenge in healthy overweight/obese subjects, and this blood glucose-lowering effect would be insulin independent.

Study 2:

1. In comparison with a CHO supplement, a CHO/AA supplement provided post-exercise will: (a) reduce the blood glucose response in healthy active subjects, and this blood glucose-lowering effect would be insulin independent, and (b) increase the rate of muscle glycogen resynthesis after a prolonged aerobic exercise bout that depletes the muscle glycogen stores.
2. In comparison with a CHO supplement, a CHO/AA supplement provided post-exercise will: (a) decrease protracted muscle damage as assessed by creatine kinase (CK) and myoglobin levels; and (b) improve the subsequent anaerobic exercise performance as assessed by a 30-sec sprint (Wingate test).
3. In comparison with a CHO supplement, a CHO/AA supplement will better phosphorylate cellular signaling proteins that control both muscle glucose uptake and glycogen synthesis.
4. The effects of the high and low AA mixtures on blood glucose homeostasis and recovery will not differ.

SIGNIFICANCE

The first study was set to test the effectiveness of an amino acid mixture, composed of isoleucine and 4 additional amino acids, in improving the blood glucose clearance of healthy overweight/obese subjects following an oral glucose tolerance test. According to data from the National Center for Health Statistics 2010 (2011), it is estimated that two-thirds of the adult population and a growing number of children in the United States are overweight or obese. Weight is considered the single strongest predictor of type 2 diabetes (Laaksonen et al., 2009), which is a metabolic disorder primarily characterized by insulin resistance and hyperglycemia.

Our laboratory recently found that gavaging Sprague-Dawley rats with this amino acid mixture improved the blood glucose response during an oral glucose challenge without an increase in the plasma insulin response (Bernard et al., 2011). The blood glucose-lowering effect of the amino acid mixture was due to an increase in skeletal muscle glucose uptake. These results suggest that our amino acid supplement acutely improves muscle insulin sensitivity and blood glucose homeostasis in rats. However, the effect of this supplement on blood glucose homeostasis has not been evaluated in humans. To our knowledge, Study 1 is the first to investigate the effect of amino acid supplementation on blood glucose homeostasis in response to an oral glucose tolerance test in humans. If we are able to show a significant improvement in glucose tolerance in overweight/obese subjects with this amino acid mixture, this mixture could be used to benefit individuals with impaired glucose tolerance and type 2 diabetics.

Study 2 continued to test the effectiveness of this amino acid mixture on the blood glucose response of healthy active subjects after an oral glucose load post prolonged aerobic exercise. Likewise, this study would be the first study to test the hypoglycemic effect of amino acids in healthy subjects. Furthermore, this study determined whether the rate of muscle glycogen resynthesis following prolonged aerobic exercise is increased with CHO/AA supplementation. This would further support the hypothesis that the amino acid mixture can improve blood clearance by increasing muscle glucose uptake and muscle glycogen synthesis in humans.

Several studies have demonstrated that carbohydrate–protein beverages can attenuate exercise-induced muscle damage (EIMD) (Baty et al., 2007; Romano-Ely et al., 2006; Saunders et al., 2004; Saunders et al., 2007). However, much less is known about the potential benefits of adding amino acids to a carbohydrate supplement post-exercise on muscle damage. Therefore, based on studies in which an anabolic response was promoted by carbohydrate-protein/amino acids supplementation post-exercise (Biolo et al., 1997; Koopman et al., 2005; Levenhagen et al., 2001; Tipton et al., 1999), we hypothesized that CHO/AA would also reduce exercise-induced muscle damage to a greater extent than CHO alone. If our hypothesis is shown to be correct, then this might be reflective of a better subsequent anaerobic exercise performance with AA/CHO supplementation.

Some studies have demonstrated that consuming a carbohydrate and protein supplement improved endurance performance in time-to-fatigue or time trial settings (Ivy et al., 2003; Saunders et al., 2004; Saunders et al., 2007) and improved muscle strength (Andersen et al., 2005; Coburn et al., 2006; Willoughby et al., 2007) when compared to a carbohydrate-only supplement. However, the effect of an amino acid mixture with carbohydrate supplement on anaerobic performance has not been investigated. If we were able to show a significant improvement in muscle glycogen replenishment, muscle tissue damage repair as well as the subsequent anaerobic exercise performance with CHO/AA supplementation, this could provide scientific evidence for coaches and trainers who recommend recovery supplements for aerobic and anaerobic athletes.

Taken together, the goals of these studies were to demonstrate more effective ways to improve blood glucose control as well as recovery from prolonged, intense endurance exercise, including increasing the rate of muscle glycogen resynthesis, reducing muscle tissue damage, and improving subsequent anaerobic exercise performance. Moreover, we tested two dosages of the amino acid mixture to determine the potency of the mixture. If we are able to show a similar effect on blood glucose control and recovery measures with CHO/LAA, this would dramatically reduce the amount of amino acids needed for the desired effects, but without the risk of possible gastrointestinal stress symptoms that have been observed with the high dosage.

LIMITATIONS AND DELIMITATIONS

These studies may have some limitations. First, large numbers of participants may not be possible due to the large time commitment by subjects and limited funding.

Second, while all effort was made to recruit subjects with similar BMI (body mass index), life style (Study 1), and fitness levels (Study 2), it is possible that there is variation in their body composition, fitness levels and training status which might affect their responses to different treatments in the study. In both studies, we required each subject to eat normally and consume his/her same diet for 48-72 hrs before each experimental trial. We also requested all the subjects to be fasting for 12 hrs before each experiment trial so that starting levels of blood glucose would be similar for all trials. We provided a nutrition drink to subjects to consume at 12 hrs before each trial during Study

1. Nevertheless, we did not control the diet throughout the course of the studies. Therefore, variations in subjects' day-to-day diets could affect their substrate metabolism during the experimental trials. We also required subjects to keep their exercise level consistent for 48-72 hrs and refrain from intense exercise for 24 hrs before each experimental trial, so that starting levels of muscle glycogen would not be affected by variations in activity or exercise performed the days before the trials. However, variations in the diet and training across the entire study period might affect the muscle metabolism during the experimental trials.

Third, both studies used a crossover design so that subjects served as their own controls. In Study 2, subjects conducted one familiarization trial, which was designed to help them be comfortable with the exercise protocol and equipment and minimize any novel components of the experimental trial. Nevertheless, a learning effect may still occur after the first experimental trial, and their experiences and perceptions in the first trial may influence their psychological approach to the second experimental trial. We randomly assigned the subjects to a sequence of different experimental treatments, which should have minimized the impact of any learning effect.

Other possible limitations lie in techniques and measurement methods. During Study 2, when taking muscle biopsies, small sample size (~50 mg) may have limitation in extrapolating to whole muscle, which might affect the accuracy to assess the muscle glycogen amount and the signaling protein activation status. For measuring creatine kinase (CK) from the serum, we collected the blood samples from subjects 24 hrs after the exercise bout for each trial. However, most studies have noted that CK levels may not increase for 2 days post-exercise and the peak values may not occur until 4 days post-exercise (Newham et al., 1986). Due to the difficulty to control the exercise level of subjects for 4 days post trials and then collect blood samples from subjects, we might not be able to observe the peak values of CK.

Our subjects were all in the age range of 20-45 (Study 1) or 19 – 35 (Study 2) who reside in Austin, Texas. Therefore, our results may not be generalizable to younger or older individuals or to other urban populations. Also, the fitness levels of subjects were selected as sedentary overweight/obese individuals for Study 1 or healthy trained athletes for Study 2, and therefore the results may not be generalizable to other populations.

During Study 2, we used cycling on a mountain bike as the mode of endurance exercise, which is composed of concentric and eccentric contractions. It is possible that substrate metabolism and muscle damage may differ from what could be found in other exercise modes. Therefore, the results of Study 2 may not be generalizable to other types of athletes or exercise.

Chapter II: Review of the Literature

Recent research suggests that the addition of protein to a carbohydrate supplement can have greater benefits on muscle metabolism and exercise recovery than supplementing carbohydrate alone. It is also reported that the components of protein, amino acids, such as leucine and isoleucine, can improve glucose tolerance in vivo and in vitro animal models by accelerating glucose uptake in peripheral tissues. Additionally, amino acids can stimulate glycogen synthesis in vitro in the absence of insulin. Our laboratory recently found that an amino acid mixture, composed of isoleucine, leucine, cysteine, methionine, and valine, improved the blood glucose response during an oral glucose challenge without an increase in the plasma insulin response in rats. Some studies have also shown that daily supplementation or acute ingestion of amino acids may prevent muscle damage that occurs as a result of prolonged intense endurance exercise or strength training and therefore improve force production and exercise performance. Therefore, in this review I will discuss the relevant literature regarding the effects of amino acids or carbohydrate/protein/amino acids supplement on glucose tolerance, muscle glycogen resynthesis, muscle damage, and the subsequent anaerobic exercise performance.

EFFECTS OF CARBOHYDRATE PLUS PROTEIN/AMINO ACIDS ON GLUCOSE TRANSPORT AND POST-EXERCISE MUSCLE GLYCOGEN RESYNTHESIS

The Amount and Timing of Post-Exercise Carbohydrate Supplementation on Muscle Glycogen Resynthesis

During intense physical training and tournament competitions, it is very important to speed up recovery in a limited time and achieve optimal performance in up coming exercise. Muscle glycogen is an important fuel source for moderate- to high-intensity exercise. It has been clearly demonstrated that aerobic endurance is directly related to the initial muscle glycogen stores (Bergstrom et al., 1967; Bergstrom and Hultman, 1966)

and that strenuous exercise cannot be maintained once these stores are depleted (Bergstrom et al., 1967; Bergstrom and Hultman, 1966; Hermansen et al., 1967). Therefore, the faster the muscle glycogen can be replenished post-exercise, the greater ability to achieve optimal performance in the subsequent exercise. In recent years, many researchers have investigated different methods of rapidly increasing muscle glycogen recovery, including type and amount of supplement to ingest (Blom et al., 1987; Burke et al., 1993; Ivy, 1998; Ivy et al., 1988b; Piehl Aulin et al., 2000; Reed et al., 1989; van Loon et al., 2000; Zawadzki et al., 1992), and the timing (Ivy et al., 1988a; Levenhagen et al., 2001) and frequency (Ivy, 1998) of supplementation. Ingestion of carbohydrate increases the blood glucose level and stimulates pancreatic insulin release, which results in a coordinated increase in glucose transport in peripheral tissues and an activation of insulin signaling for glycogen synthesis. It has been suggested that the rate of muscle glycogen synthesis post-exercise is maximized when a carbohydrate supplement in excess of 1.0 g/kg body weight is consumed immediately post-exercise (Ivy, 1998; Ivy et al., 1988a; Levenhagen et al., 2001). The rapid rate of glycogen recovery can be maintained up to six hours post-exercise when the carbohydrate supplement is ingested at two hour intervals (Ivy, 1998). Some studies demonstrated that the rate of glycogen resynthesis post-exercise could be further increased when the supplement is provided at a rate of ~0.25 to 0.4 g CHO/kg every 15 min or ~1.0 g CHO/kg every 30 min (total ~2.0 to 4.2g CHO/kg/2hr) (Doyle et al., 1993; Piehl Aulin et al., 2000; van Hall et al., 2000). However, the amount of CHO they used is too large to consume post-exercise, especially for those who must maintain body weight within a weight class and who are exercising to reduce weight. The frequent ingestion rate (every 15-30 min) is also not practical for most athletes, especially during limited recovery intervals between intense exercise bouts. Therefore, the search for means to reduce the carbohydrate content of a sports drink, while maintain or even enhance performance is desired.

Effects of Carbohydrate/Protein Supplementation on Post-Exercise Muscle Glycogen Resynthesis

Recent studies suggested that the inclusion of small amounts of protein in a carbohydrate-electrolyte beverage may enhance muscle glycogen recovery post-exercise

over traditional carbohydrate-only beverages (Berardi et al., 2006; Ivy et al., 2002; van Loon et al., 2000; Zawadzki et al., 1992). This has been credited, in part, to the ability of protein and carbohydrate to act synergistically on insulin secretion (van Loon et al., 2000; Zawadzki et al., 1992). In a study by Zawadzki et al. (Zawadzki et al., 1992), carbohydrate (112g) or carbohydrate (112g) plus protein (40.7g) was provided immediately and 2 hours after exercise. It was found that adding protein to a carbohydrate supplement caused a greater insulin response, a significantly lower plasma glucose response and a faster rate of muscle glycogen recovery, compared with a carbohydrate treatment of similar amount.

Several subsequent studies ascribed the benefit of carbohydrate-protein supplementation to the greater calorie content contained in the feedings. These studies with isocaloric supplementation showed no extra effect of carbohydrate-protein on muscle glycogen recovery relative to carbohydrate-only supplementation (Carrithers et al., 2000; Jentjens et al., 2001; van Loon et al., 2000). However, a more recent research employing natural abundance ^{13}C-nuclear magnetic resonance (NMR) spectroscopy to measure muscle glycogen confirmed that the muscle glycogen resynthesis rate is enhanced when a carbohydrate-protein supplement is consumed post-exercise, compared to an isocaloric carbohydrate-only supplement (Berardi et al., 2006; Ivy et al., 2002). ^{13}C-NMR spectroscopy is a noninvasive, highly sensitive technique, providing continuous and more precise measurement of muscle glycogen content compared with the muscle biopsy technique (Price et al., 1999). The use of the noninvasive ^{13}C-NMR spectroscopy technique might be one important factor for detecting a statistical difference in glycogen storage compared with studies using the muscle biopsy technique.

There are other differences in the methodologies of these studies that may account for the conflicting findings. Different amount of macronutrients and the timing/frequency of supplementation provided during the recovery period might be one possibility. The ability of protein to improve glycogen resynthesis rate might be restricted to the conditions when protein is added to low or moderate amounts of carbohydrate (0.6-0.8 g/kg/h) and provided at intervals of 1-2 hours or greater (Berardi et al., 2006; Ivy et al., 2002). The previous studies, which found no additional effect of isocaloric carbohydrate-protein supplements on glycogen resynthesis, gave their subjects large amount of CHO

(1.0-1.2g/kg/h) at more frequent intervals (every 30min) (Carrithers et al., 2000; Jentjens et al., 2001; van Loon et al., 2000). The large amount of carbohydrate mixed with protein might have altered the absorption rate of carbohydrate and protein and possibly limited the advantage of adding protein to a carbohydrate supplement.

Another possible reason for the conflicting findings in these studies may have been the exercise protocol used to deplete the muscle glycogen. It was indicated that insulin-independent glucose uptake and glycogen synthesis happens when glycogen concentrations are lower than 120-150 mmol/kg dry muscle (Berardi et al., 2006; Price et al., 1999). Therefore, when the glycogen concentration is below this threshold level, there is a high physiological drive for muscle glucose uptake and muscle glycogen resynthesis independent of insulin (Berardi et al., 2006). Previous studies which found no effect of isocaloric carbohydrate-protein supplementation on glycogen resynthesis employed strenuous exercise protocols, which resulted in a very low post-exercise muscle glycogen content (~100mmol/kg dry muscle) (Berardi et al., 2006; Carrithers et al., 2000; Ivy et al., 2002; Jentjens et al., 2001; van Loon et al., 2000). In these studies, CHO supplement alone might be sufficient to stimulate maximal muscle glycogen replenish rate. The more recent studies using ^{13}C-NMR spectroscopy employed less strenuous protocols, which resulted in moderate extent of muscle glycogen depletion and post-exercise concentrations are ~160-200mmol/kg dry muscle. They found enhanced muscle glycogen storage when adding protein to a carbohydrate supplement (Berardi et al., 2006; Ivy et al., 2002). Thus, the severe exercise protocol might conceal the benefit of protein added to a carbohydrate supplement.

The Signaling Pathway Underlying the Effects of Carbohydrate plus Protein on Post-Exercise Muscle Glycogen Resynthesis

The underlying signaling pathway responsible for the beneficial effect of CHO-PRO on muscle glycogen resynthesis was investigated recently (Fig. 2.1). Ivy et al. (Ivy et al., 2008) found that a CHO-PRO supplement post-exercise alters the phosphorylation levels of the enzymes involved in muscle glycogen synthesis, like protein kinase B (Akt/PKB), the mammalian target of rapamycin (mTOR), ribosomal protein S6 (rpS6), glycogen synthase kinase 3 (GSK-3) and glycogen synthase (GS). Another study from

our laboratory also demonstrated in the rat that post-exercise CHO-PRO supplementation was more effective in activating the mTOR-dependent signaling pathway, especially rpS6 and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) in red and white skeletal muscles, compared to either CHO or PRO supplementation (Morrison et al., 2008). More recently, we investigated the effects of cereal with nonfat milk (Cereal) post prolonged exercise on muscle glycogen resynthesis and associated enzyme activation, compared with a traditional carbohydrate-electrolyte sports drink (Drink) (Kammer et al., 2009). Plasma insulin was significantly increased after Cereal although plasma glucose was similar between treatments. Muscle signaling proteins, like mTOR, GS, and Akt/PKB, were significantly activated during recovery after Cereal. Cereal also blunted the increase in plasma lactate post-exercise, which suggests that a higher percentage of glucose transported into muscle was converted to muscle glycogen after Cereal.

Effects of Amino Acids on Muscle Glycogen Synthesis and the Underlying Signaling Pathway

The benefits of addition of protein to carbohydrate supplement post-exercise have been widely studied in recent years. However, whether the components of protein, amino acids, have a similar effect on muscle recovery is not clear. Physiological concentrations of amino acids in vitro have been found to stimulate glycogen synthesis directly in the absence of insulin (Armstrong et al., 2001; Peyrollier et al., 2000). It was suggested that amino acids, specially leucine, in vitro stimulate glycogen synthesis via activating mTOR and/or the 70 kDa ribosomal S6 kinase (p70S6K) in the absence of insulin, without paralleled activation of Akt/PKB (Fig. 2.1) (Armstrong et al., 2001; Peyrollier et al., 2000). It was presumed that activated p70S6K might induce transient inactivation of GSK-3, which triggers the initial activation of GS, and amino acids could further maintain the activation of GS via the rapamycin-sensitive pathway without the involvement of GSK-3 (Armstrong et al., 2001). However, the effects of amino acids in vivo on GSK-3 and/or GS activities are not obviously like that of insulin, partly due to the fact that insulin also can stimulate Akt/PKB phosphorylation and subsequently phosphorylates GSK-3 via a p70S6K-independent mechanism.

A study by Ruby et al. (Ruby et al., 2005) found that the addition of 4-hydroxyisoleucine (4-OH-Ile), an amino acid extract purified from fenugreek seeds, to a carbohydrate supplement has been found to increase muscle glycogen synthesis post-exercise. Research indicated that 4-OH-Ile, combined with a moderate carbohydrate supplement (0.9 g/kg/h) and provided immediately and 2 hours after exercise in trained male cyclists, was able to stimulate greater post-exercise muscle glycogen synthesis compared with carbohydrate-only without any difference in plasma insulin concentration between the two trials (Ruby et al., 2005). These results suggest that 4-OH-Ile in combination with a moderate amount of carbohydrate might improve the rate of muscle glycogen synthesis post-exercise via an alternative mechanism independent of an enhanced plasma insulin response. However, the underlying signaling pathway for the effect of 4-OH-Ile on muscle glycogen synthesis is not clear.

Effects of Amino Acids Alone or Combined with Carbohydrate on Glucose Transport and the Underlying Signaling Pathway

Amino acids might also enhance glycogen synthesis indirectly by stimulating glucose transport. Several studies have reported that leucine and/or isoleucine might stimulate glucose transport independently of insulin/Akt/PKB, possibly via an mTOR-independent phosphatidylinositol 3-kinases (PI3K)/ atypical isoforms of protein kinase C (aPKC) pathway (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007; Nishitani et al., 2002). Nashitani et.al. (Nishitani et al., 2002) reported that 1ml of 2mM leucine promoted glucose transport in isolated rat soleus muscles (0.1-0.2g). The leucine-stimulated glucose transport in rat muscle was maintained at high rates from 15-45 minutes and declined thereafter. This stimulating effect on glucose transport of leucine was suppressed by 10 μM LY294002 or 6 μM GF109203X, which are specific inhibitors for PI3K and aPKC, respectively (Uberall et al., 1999). PI3K is essential for insulin-regulated GLUT4 translocation and glucose uptake (Clarke et al., 1994; Haruta et al., 1995; Katagiri et al., 1996; Kotani et al., 1995; Tanti et al., 1996; Thong et al., 2005). aPKCs are downstream proteins of insulin receptor substrate (IRS)/PI3K and have been suggested to be able to increase glucose transport in skeletal muscle by vesicle-associated membrane protein 2 (VAMP2) serine phosphorylation in the glucose transporter type 4

(GLUT-4) compartment (Nishitani et al., 2002; Thong et al., 2005). It is known that leucine has no effect on Akt/PKB (Greiwe et al., 2001), which is another downstream protein of IRS/PI3K and contributes to insulin regulation of GLUT4 traffic and glucose uptake (Thong et al., 2005). Furthermore, pre-treatment of 1 μM rapamycin did not significantly affect increased glucose transport by leucine and this study was conducted under insulin-free conditions. Taken together, leucine might stimulate glucose transport differently from insulin, possibly via an mTOR-independent PI3K/aPKC pathway.

However, the combination of carbohydrate and leucine appears to have no additive effect on glucose transport. Baum et al. (Baum et al., 2005) evaluated the impact of a physiological oral dose of leucine and/or carbohydrate on glucose uptake in rats. Rats were fed oral doses of carbohydrate (CHO) (1.31g glucose, 1.31g sucrose), leucine (270mg), or CHO plus leucine (CHO/Leu) after 12 hours fasting. It was shown that during the first 60 minutes after oral administration, CHO/Leu did not cause any difference in glucose uptake when compared with CHO alone. However, the activity of PI3K in skeletal muscle was suppressed at 30, 60, and 90 minutes following CHO/Leu supplement. It was proposed that the decreased PI3K activity while maintaining normal levels of glucose transport might be due to increased degradation of the IRS1/PI3K complex rather than inhibition of the IRS1/PI3K complex formation. Therefore, the initiated signal is still able to activate physiological events, such as glucose transport. The blunting effect on the IRS1/PI3K pathway may result from the overactivation of mTOR/p70S6K by leucine, which might serves as a component of the feedback regulation of PI3K signaling pathway (Baum et al., 2005; Iwanaka et al., 2010; Tremblay et al., 2005a; Tremblay and Marette, 2001).

Isoleucine has also been found to increase glucose uptake in an insulin-independent manner (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007). In an oral glucose tolerance test in normal rats, a single dose of 0.3 g/kg body wt isoleucine significantly reduced plasma glucose levels 30 and 60 min after the glucose bolus (Doi et al., 2003). In an in vivo study in food-deprived rats, 1.35 g/kg body wt isoleucine significantly increased skeletal muscle glucose uptake (Doi et al., 2005). In the absence of insulin, 1 mM isoleucine also significantly increased glucose consumption in C_1C_2 myotubes (Doi et al., 2003). The signaling pathway analysis using several inhibitors

found that the potent hypoglycemia effect of isoleucine *in vitro* was mediated by PI3K and aPKC, which was independent of mTOR, similar to leucine-mediated glucose uptake in isolated muscle (Nishitani et al., 2002). It is also found that glucose uptake by skeletal muscle might be the major mechanism by which isoleucine lowers blood glucose levels in vivo (Doi et al., 2005; Doi et al., 2007).

It has been found that isoleucine has no effects on mTOR/p70S6K (Atherton et al., 2010). Therefore, the addition of isoleucine to carbohydrate supplement might be more efficient on glucose transport than carbohydrate/leucine combination. Our laboratory recently found that gavaging both normal Sprague-Dawley rats and insulin-resistant obese Zucker rats with an amino acid mixture, composed of isoleucine and 4 other amino acids, improved the blood glucose response during an oral glucose challenge without an increase in the plasma insulin response (Bernard et al., 2011). We also found that the amino acid mixture significantly elevated phosphorylation of Akt substrate of 160 kDa (AS160) and markedly decreased GS phosphorylation without altering Akt/PKB activation stimulated by carbohydrate in Sprague-Dawley rat skeletal muscles. AS160 is one potential candidate of downstream targets of Akt/PKB and has been suggested to regulate GLUT-4 translocation in response to either insulin or contractile activity (Bruss et al., 2005; Cheng et al., 2005; Eguez et al., 2005; Kane et al., 2002). Another recent *in vitro* study from our laboratory also found that an amino acid mixture increases glucose uptake in isolated rat epitrochlearis muscle in the absence of insulin (Kleinert et al., 2011). This effect is associated with an increased phosphorylation of AS160 without significant changes in mTOR or Akt/PKB. These results suggested that the amino acid mixture improves glucose tolerance by increasing skeletal muscle glucose uptake in rats through an as yet undefined intracellular signaling cascade (Fig.2.1).

The findings of the many studies discussed above indicate that combined ingestion of protein/amino acids and carbohydrate further increases the rate and amount of muscle glycogen resynthesis when taken immediately and 1-2 hours post-exercise. This would be more practical for those who have only limited time to recovery between training sessions or competitive events and would be beneficial for those who must limit their carbohydrate intake and maintain or reduce body weight while maintaining or even

enhancing performance. The potential hypoglycemic effect of amino acids, especially isoleucine, might be also beneficial for those glucose intolerant individuals.

EFFECTS OF CARBOHYDRATE PLUS PROTEIN/BCAA ON MUSCLE DAMAGE AND THE SUBSEQUENT EXERCISE PERFORMANCE

Following prolonged strenuous exercise, the body is in a catabolic state in which blood insulin is low and the catecholamines and cortisol are high. Although there is an increase in muscle protein synthesis, its rate is exceeded by the rate of protein degradation such that there is a negative net protein balance. The ingestion of carbohydrate and protein following exercise has been reported to promote protein synthesis and hastens recovery (Koopman et al., 2005; Levenhagen et al., 2001).

Supplementation with a mixture of essential amino acids will also increase protein synthesis and suppress protein degradation (Biolo et al., 1997; Blomstrand and Newsholme, 1992; Tipton et al., 1999). Blomstrand et al. (Blomstrand and Newsholme, 1992) supplied BCAA to well-trained endurance athletes during two types of sustained, intensive running exercise, a 30 km cross-country race and a full marathon. The total amount of BCAA ingested by each subject during the cross-country race was 7.5g and composed of 35% leucine, 50% valine, and 15% isoleucine. During the full marathon, each subject ingested 12g BCAA, which was composed of 35% leucine, 40% valine, and 25% isoleucine. In the placebo group, the amount of the aromatic amino acids, tyrosine and phenylalanine, in the muscle was increased by 20-40% after both types of exercise, and the plasma concentration of these amino acids was increased after the marathon. However, when BCAA were supplied during both types of exercise, the increases in tyrosine and phenylalanine concentrations in both plasma and muscle were inhibited. Since tyrosine and phenylalanine are neither taken up nor metabolized by skeletal muscle, the increased concentrations of them in muscle might indicate net protein degradation during exercise. Therefore, these results suggest that BCAA supplementation during exercise may prevent or decrease the net rate of protein degradation caused by prolonged intense exercise.

Furthermore, adding amino acids to a carbohydrate supplement could provide an efficient intervention to optimize net skeletal muscle protein balance during post-exercise

recovery (Koopman et al., 2005), taking advantage of an enhanced insulin response, energy availability, and substrate for protein synthesis. In addition, Miller et al. (Miller et al., 2003) evaluated the combined effects of essential amino acids and carbohydrate administered after resistance exercise on muscle protein metabolism. Subjects ingested one of three drinks (~6 g amino acids (AA), ~35 g carbohydrate (CHO), or AA and CHO (MIX)) at 1 and 2 h after leg resistance exercise. Total net uptake of phenylalanine across the leg over 3 h was greatest in MIX and least in CHO, which was due to increased muscle protein synthesis in MIX. The results showed that the combined effects of amino acids and carbohydrate supplementation on muscle protein synthesis are additive, and roughly equivalent to the sum of the independent effects of either given alone.

These studies indicate that carbohydrate-protein/amino acids supplementation promoted the anabolic response and inhibited the catabolic response post exercise, resulting in a positive net protein balance. Improved protein balance might result in a protection or a faster repair of muscle damage. It could be interesting to know whether carbohydrate-protein/amino acids supplementation would also reduce exercise-induced muscle damage (EIMD).

Exercise-induced Muscle Damage and its Effects on Exercise Performance

EIMD occurs following either unaccustomed physical activity, or activity of great intensity or duration. Many studies reported that muscle damage mainly occurred in exercise involving a large amount of eccentric muscle contractions, such as plyometric jumps (Marginson et al., 2005), downhill backward walking (Nottle and Nosaka, 2005), marathon running (Asp et al., 1999; Asp et al., 1997; Tuominen et al., 1996), resistance training (Baty et al., 2007), and bench stepping (Vickers, 2001). This damage is mainly associated with damage to the excitation-contraction coupling system and disruption at the level of the sarcomeres (Byrne et al., 2004; Clarkson and Hubal, 2002; Proske and Morgan, 2001). Other studies, however, reported that muscle damage also occurs in prolonged intense exercise involving concentric actions such as cycling but in a lesser degree (Bessa et al., 2008; Greer et al., 2007; Koller et al., 1998; Millet and Lepers, 2004; Romano-Ely et al., 2006; Saunders et al., 2004; Saunders et al., 2007).

EIMD does not just occur during exercise, but can continue after exercise for many hours. Muscle damage could cause an immediate and prolonged reduction in muscle function, including reductions in muscle force production and power generation. It was suggested that the measurement of muscle function provides the most effective means of evaluating the magnitude and time course of damage caused by exercise (Warren et al., 1999). A number of studies have quantified the declines in isometric strength (Avela et al., 1999; Byrne and Eston, 2002; Clarkson et al., 1992; Howell et al., 1993; Komi and Viitasalo, 1977; Sayers and Clarkson, 2001), isokinetic concentric and eccentric strength (Byrne et al., 2001; Deschenes et al., 2000; Michaut et al., 2002), vertical jump performance (Avela et al., 1999; Byrne and Eston, 2002; Horita et al., 1999), and endurance performance (Gleeson et al., 1998; Gleeson et al., 1995). However, few studies have examined the effect of muscle damage on power generation. Muscular power is the product of force and velocity. Therefore, a similar reduction in power and strength resulting from muscle damage would be expected. Sherman et al. (Sherman et al., 1984) employed a maximal work capacity test consisting of 50 maximal leg extensions at 3.2 rad/sec and found 47% reduction in work capacity immediately after a marathon race. Byrne et al. (Byrne and Eston, 2002) reported immediate and prolonged reductions (up to 3 days) in dynamic peak power during a 30-second Wingate cycle test after 100 repetitions of the eccentric phase of the barbell squat exercise. The Wingate cycle test has been shown to be a highly reliable and applicable test for predicting performance in both individual and team sporting events (Nottle and Nosaka, 2007; Sinnett et al., 2001). Because the external load during the Wingate test remains constant, a reduction in Wingate peak power is the direct result of an inability to achieve a high pedal frequency against a constant resistance. Another study by Nottle et al. (Nottle and Nosaka, 2007) also reported an immediate reduction in peak and average power during the 30-second Wingate test following a 40-minute down hill run on a treadmill.

Effects of Carbohydrate plus Protein/Amino Acids Supplementation on Muscle Damage and the Subsequent Exercise Performance

Carbohydrate–protein beverages have been reported to attenuate exercise-induced muscle damage and maintain exercise performance (Baty et al., 2007; Romano-Ely et al.,

2006; Rowlands et al., 2008; Saunders et al., 2004; Saunders et al., 2007). A recent study from our lab found that ingestion of a carbohydrate-protein supplement before and during a resistance exercise session significantly reduced muscle damage, as evidenced by decreased responses of both plasma myoglobin and creatine kinase (CK), which are blood markers of muscle damage (Baty et al., 2007). Saunders et al. (Saunders et al., 2004; Saunders et al., 2007) reported that ingestion of a carbohydrate-protein supplement, in both beverage- and gel-forms, during and after exercise significantly reduced muscle damage in endurance cyclists, accompanied with lower plasma CK concentration. Moreover, these cyclists rode 13%-29% longer in timed cycle-trials at 75% VO_2 peak to volitional exhaustion when consuming CHO/PRO beverage or gel during exercise than CHO only beverage. They also performed 40% longer in a second ride to exhaustion at 85%VO_2 peak at 12-15h later when consuming CHO/PRO beverage than consuming CHO only beverage.

In recent years, some studies have shown that BCAA supplementation are beneficial for reducing EIMD and fatigue, maintaining muscle force and exercise performance (Blomstrand and Newsholme, 1992; Coombes and McNaughton, 2000; Greer et al., 2007; Jackman et al., 2010; Negro et al., 2008; Shimomura et al., 2010; Skillen et al., 2008). The effects of BCAA supplementation on serum indicators of muscle damage after prolonged exercise were investigated in a study by Coombes et al. (Coombes and McNaughton, 2000). Sixteen male subjects took 12 g/day BCAA in addition to their normal diet or only took the normal diet for 14 days. There was no difference in the baseline serum CK and lactate dehydrogenase (LDH), both of which are shown to be accurate indicators of muscle damage. On day 7, subjects cycled on an ergometer at ~70% VO2max for 2 hrs. Both serum CK and LDH concentrations were significantly increased thereafter until 5 days post-exercise in the placebo group, while the BCAA supplementation significantly reduced the increase in serum LDH from 2 hrs to 5 days, and serum CK from 4 hrs to 5 days post-exercise. These results indicate that daily supplementation of BCAA may reduce the serum concentrations of intramuscular enzymes CK and LDH associated with EIMD.

Shimomura et al. (Shimomura et al., 2010) examined the acute effect of three BCAA supplementation (isoleucine:leucine:valine = 1:2.3:1.2) taken before exercise on

muscle damage. The subjects ingested BCAA or dextrin at 100 mg/kg body weight before a series of squat exercise, which consisted of 7 sets of 20 squats/set with 3-min intervals between sets. Serum myoglobin concentration was increased post-exercise in the placebo trial but not in the BCAA trial. In both trials, exercise-induced delayed-onset muscle soreness (DOMS) showed a peak on Day 2 and 3, but the level of soreness was significantly lower in the BCAA trial compared with the placebo trial. Leg-muscle force during maximal voluntary isometric contractions was significantly decreased on Day 2 in the placebo trial but not in the BCAA trial. These results suggest that BCAA supplementation before strength training exercise may suppress EIMD and maintain muscle force.

The effect of BCAA supplementation on muscle damage caused by endurance exercise involving concentric actions was investigated in a study by Greer et al. (Greer et al., 2007). Nine untrained subjects cycled at 55% VO2peak for 90 min and consumed BCAA or CHO beverage before and at 60 min of exercise. CK activities and ratings of perceived soreness were significantly lower during the BCAA trial than the CHO trial at 24 h post-exercise. Leg-flexion torque was higher during the BCAA trial at 48 h post-exercise. These results suggest that BCAA supplementation attenuates muscle damage and prevents muscle torque drop post prolonged concentric exercise.

Furthermore, Skillen et al. (Skillen et al., 2008) examined the effect of amino acids in a carbohydrate beverage on muscle damage and cycling performance. Twelve male athletes took 3.6% carbohydrate plus 1% amino acids (AA) or 4.6% carbohydrate-only (CHO) beverages for 2 weeks. They cycled at 75% VO2 peak for 90 min followed by a ride to exhaustion at 85% VO2 peak before (T1) and on 2 consecutive days (T2 and T3) after 2 weeks of supplementation. Plasma CK levels were significantly lower for AA in T3. Time to exhaustion decreased from T2 to T3 only in CHO but not in AA. Changes in vertical-jump pre- to post-exercise were greater in T3 for the CHO treatment. Supplementation with AA significantly decreased total fatigue score and mood disturbance in T3. These data suggest that the addition of AA to a carbohydrate beverage reduced muscle damage as indicated by CK levels, decreased muscle fatigue, and maintained the subsequent endurance exercise performance compared with consuming carbohydrate alone.

The findings of the studies discussed above indicate that daily supplementation or acute ingestion of protein and/or BCAA may prevent muscle damage that occurs as a result of a prolonged, intense endurance exercise or strength training. Moreover, some studies have demonstrated that consuming protein/amino acids supplement improved muscle strength when compared to a carbohydrate only supplement (Andersen et al., 2005; Coburn et al., 2006; Shimomura et al., 2010; Willoughby et al., 2007). Furthermore, consuming the combined supplement of carbohydrate and protein has been shown to improve endurance performance in time-to-fatigue or time trial settings (Ivy et al., 2003; Saunders et al., 2004; Saunders et al., 2007). The effects of amino acids in a carbohydrate beverage on muscle damage and cycling performance have also been examined in a study by Skillen et al. (Skillen et al., 2008). However, much less is known about the effect of carbohydrate plus amino acids supplement on anaerobic exercise performance. Based on the fact that amino acids have been found to prevent exercise-induced muscle damage, it is reasonable to hypothesize that supplementing post-exercise with carbohydrate plus amino acids will lead to a faster post-exercise recovery and improve subsequent anaerobic exercise performance.

CONCLUSION

During intense physical training and tournament competitions, it is very important to speed up recovery when time is limited to achieve optimal performance in subsequent exercise. Recovery from exercise involves the integration of multiple systems and many components which are directly affected by post-exercise nutrition. Compared to carbohydrate supplements alone, carbohydrate plus protein/amino acids further increased the rate and amount of muscle glycogen resynthesis when taken immediately and 1-2 hours post-exercise. This provides a more practical method for those who have only limited time to recovery and those who must limit their carbohydrate intake and maintain or reduce body weight while maintaining or even enhancing performance. The components of protein, amino acids, have also been shown to stimulate glycogen synthesis via activating mTOR and/or p70S6K in the absence of insulin. Amino acids might also enhance glycogen synthesis indirectly by stimulating glucose transport via an mTOR-independent PI3K/aPKC pathway. Moreover, the potential hypoglycemic effect

of amino acids, especially isoleucine, might be also beneficial for those insulin intolerant individuals. In addition, protein and/or BCAA supplements may prevent exercise-induced muscle damage and maintain muscle strength, compared to a carbohydrate only supplement. The combination of carbohydrate and protein/amino acids has been shown to improve endurance performance in time-to-fatigue or time trial settings, but much less is known about anaerobic exercise performance. A summary for the previous studies about the effects of protein/amino acids supplementation on glucose metabolism and muscle damage is listed in Table 2.1. While it has not previously been investigated, we hypothesize that a carbohydrate plus amino acid mixture supplement can lower blood glucose levels, increase muscle glycogen resynthesis, suppress exercise-induced muscle damage post-exercise, and improve subsequent anaerobic exercise performance.

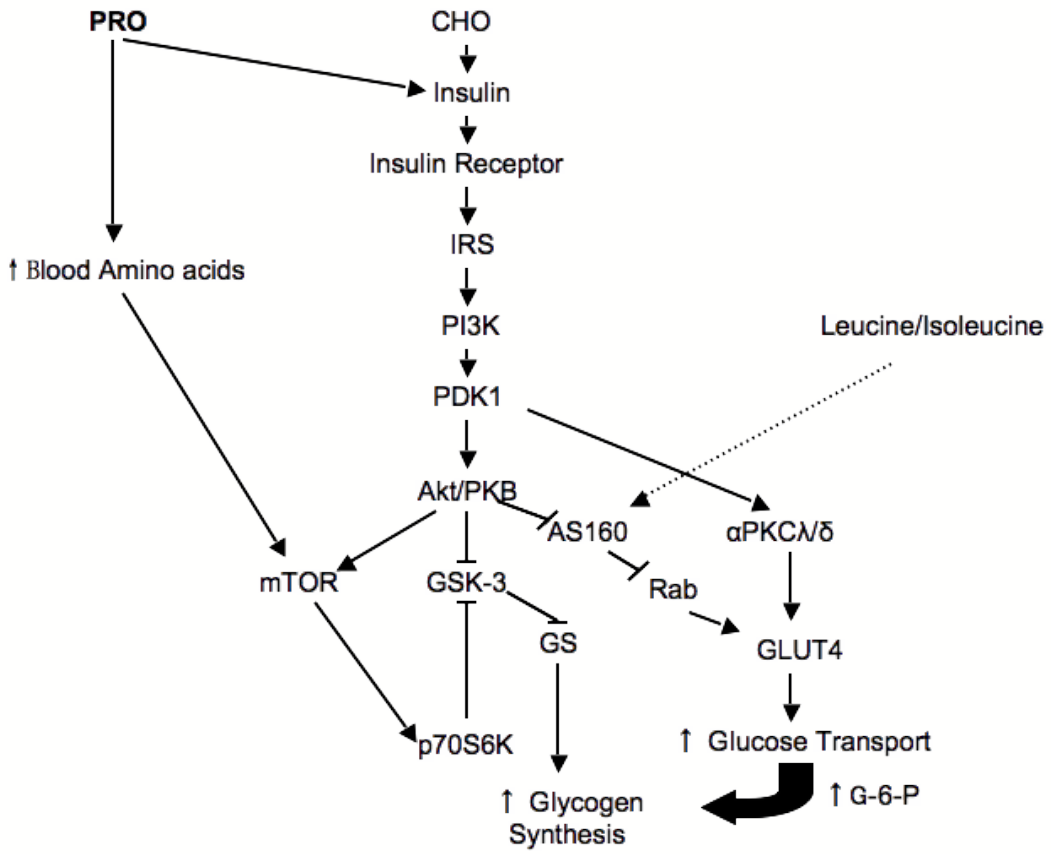


FIGURE 2.1. INSULIN AND MTOR SIGNALING PATHWAYS IN GLUCOSE TRANSPORT AND MUSCLE GLYCOGEN SYNTHESIS. ——►, ·····►, Activating steps; —┤, inhibitory steps in the pathway. ·····, Incompletely-defined links. CHO, carbohydrate; PRO, protein; IRS, insulin receptor substrates; PDK, phosphatidylinositol phosphate-dependent protein kinase; Akt/PKB, protein kinase B. AS160, Akt substrate of 160kDa; αPKC-λ/δ, atypical protein kinase C; GSK-3, glycogen synthase kinase-3; GS, glycogen synthase; p70S6K, 70kDa ribosomal protein (rp) S6 kinase; See text for further details.

Study	Subjects	Supplementation	Amount	Time	Blood Glucose Level	Blood Insulin Level	Glucose Uptake	Muscle Glycogen Synthesis	Muscle Damage	Muscle degradation
Zawadzki et al. 1992	male cyclists	CHO-PRO	112 g CHO + 40.7 g PRO	immediately and 2 hrs post exercise	decrease	increase	N/A	increase	N/A	N/A
Ivy et al. 2002	male cyclists	CHO-PRO	80 g CHO + 28 g PRO	immediately and 2 hrs post exercise	decrease	no change	N/A	increase	N/A	N/A
van Loon et al. 2000	trained cyclists	CHO-PRO-Leu-Phe	0.8 g CHO + 0.4 g PRO/AA/kg body wt./h	every 30 min until 270 min post exercise	N/A	increase	N/A	increase	N/A	N/A
Hara et al. 2011	rats	CHO-PRO	0.9 g CHO + 0.3 g PRO/kg body wt.	N/A	N/A	N/A	N/A	increase	N/A	N/A
Armstrong et al. 2001	human muscle cell culture	amino acids	See reference	N/A	N/A	N/A	N/A	increase	N/A	N/A
Ruby et al. 2005	male cyclists	4-hydroxy-isoleucine	1.8 g CHO + 2 mg 4-OH-Ile/kg body wt.	immediately and 2 hrs post exercise	no change	no change	N/A	increase 63%	N/A	N/A
Nishitani et al. 2002	isolated soleus muscle	leucine	2 mM	N/A	N/A	N/A	increase	N/A	N/A	N/A
	isolated muscle	a-ketoisocaproic acid	2 mM	N/A	N/A	N/A	increase	N/A	N/A	N/A
Doi et al. 2003	rats	isoleucine	0.3 g/kg body wt.	30 min before OGTT	decrease	decrease	N/A	N/A	N/A	N/A
	rats	leucine	0.3 g/kg body wt.	30 min before OGTT	increase	decrease	N/A	N/A	N/A	N/A
	rats	valine	0.3 g/kg body wt.	30 min before OGTT	increase	decrease	N/A	N/A	N/A	N/A

	C_2C_{12} myotubes	isoleucine	1 mM	N/A	N/A	N/A	increase 16.8%	no change	N/A	N/A
	C2C12 myotubes	leucine	1 mM	N/A	N/A	N/A	no change	increase	N/A	N/A
	C2C12 myotubes	valine	1 mM	N/A	N/A	N/A	no change	increase	N/A	N/A
Doi et al. 2005	rats	isoleucine	1.35 g/kg body wt.	N/A	decrease	no change	increase 73%	no change	N/A	N/A
	rats	leucine	1.35 g/kg body wt.	N/A	no change	no change	no change	increase	N/A	N/A
Doi et al. 2007	rats	isoleucine	0.45 g/kg body wt.	N/A	decrease 20%	no change	increase 71%	N/A	N/A	N/A
	rats	leucine	0.45 g/kg body wt.	N/A	no change	increase	no change	N/A	N/A	N/A
Ikehara et al. 2008	normal mice	isoleucine	300 mg/kg body wt.	N/A	decrease	no change	N/A	N/A	N/A	N/A
	glucose-intolerant mice	isoleucine	300 mg/kg body wt.	N/A	decrease	increase	N/A	N/A	N/A	N/A
	Type 2 diabetes mice	isoleucine	500 mg/kg body wt.	N/A	decrease	no change	N/A	N/A	N/A	N/A
Morifuji et al. 2008	L6 myotubes	isoleucine-leucine	1 mM	N/A	N/A	N/A	increase	increase	N/A	N/A
Bernard et al. 2011	rats	CHO-AA mixture	see reference	N/A	decrease	no change	increase	N/A	N/A	N/A
Kleinert et al 2011	isolated muscle	AA mixture	see reference	N/A	N/A	N/A	increase	N/A	N/A	N/A
Kalogeropo-ulou et al. 2008	healthy adults	leucine	1 mmol/kg lean body mass	N/A	no change	increase	N/A	N/A	N/A	N/A
		leucine/glucose	1 mmol/kg lean body mass + glucose 25 g	N/A	decrease 50%	increase	N/A	N/A	N/A	N/A

Nuttal et al. 2008	healthy adults	isoleucine	1 mmol/kg lean body mass	N/A	decrease	no change	N/A	N/A	N/A	N/A
		Isoleucine /glucose	1 mmol/kg lean body mass + 25 g glucose	N/A	decrease	increase	N/A	N/A	N/A	N/A
Baty et al. 2007	male	CHO-PRO vs. Placebo	6.2% CHO + 1.5% PRO	before, during and post exercise	N/A	increase	N/A	N/A	decrease	N/A
Saunders et al. 2004	male cyclists	CHO-PRO	7.3% CHO + 1.8% PRO	every 15 min during exercise and immediately post exercise	N/A	N/A	N/A	N/A	decrease	N/A
Coombes et al. 2000	male adults	BCAA	12g per day	14 days	N/A	N/A	N/A	N/A	decrease	N/A
Greer et al. 2007	untrained men	BCAA	200 kcal of energy via the CHO or BCAA beverage	before and at 60 min of exercise	N/A	N/A	N/A	N/A	decrease	N/A
Shimomura et al. 2010	untrained females	BCAA	100 mg/kg body wt.	before exercise	N/A	N/A	N/A	N/A	decrease	N/A
Skillen et al. 2008	male athletes	CHO-BCAA	3.6% CHO + 1% BCAA per day	for 2 weeks	N/A	N/A	N/A	N/A	decrease	N/A
Blomstrand et al. 1992	trained athletes	BCAA	7.5-12 g	during exercise	N/A	N/A	N/A	N/A	N/A	decrease

Table 2.1 Summary for the previous studies about the effects of protein/amino acids on glucose metabolism and muscle damage.

Chapter III: Amino Acid Mixture Acutely Improves the Glucose Tolerance of Healthy Overweight/Obese Adults

ABSTRACT

Certain amino acids have been reported to influence carbohydrate metabolism and blood glucose clearance, as well as improve the glucose tolerance in animal models. The aim of the present study was to investigate the effect of an amino acid mixture on the glucose response of healthy overweight/obese men and women to an oral glucose tolerance test (OGTT). Twenty-two overweight/obese healthy subjects completed two OGTT after consuming two different test beverages. The amino acid mixture beverage (CHO/AA) consisted of 0.088 g cystine 2HCl, 0.043 g methionine, 0.086 g valine, 12.094 g isoleucine, 0.084 g leucine and 100 g dextrose. The control beverage (CHO) consisted of 100 g dextrose, only. Venous blood samples were drawn 10 min before the start of ingesting the drinks, and 15, 30, 60, 120 and 180 min after the completion of the drinks. During the OGTT, the plasma glucose response for the CHO/AA treatment was significantly lower than that of the CHO treatment, as was the plasma glucose area under the curve (CHO/AA 806 ± 31 mmol/L•3h vs. CHO 942 ± 40 mmol/L•3h). Differences in plasma glucose between treatments occurred at 30, 60, 120, and 180 min after supplement ingestion. Plasma glucagon during the CHO/AA treatment was significantly higher than during the CHO treatment. However, there were no significant differences in plasma insulin or c-peptide responses between treatments. These results suggest that our amino acid mixture lowers the glucose response to an OGTT in healthy overweight/obese subjects in an insulin-independent manner.

INTRODUCTION

Type 2 diabetes is a metabolic disorder that is primarily characterized by insulin resistance and hyperglycemia. Skeletal muscle is the predominate tissue for the clearance of blood glucose and 70 to 80% of an oral glucose challenge is cleared by skeletal muscle (DeFronzo et al., 1981; Katz et al., 1983; Willems et al., 1991). Recent research has demonstrated that certain amino acids, such as leucine and isoleucine, can improve blood

glucose clearance and uptake in skeletal muscles *in vivo* and *in vitro* (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007; Kalogeropoulou et al., 2008; Morifuji et al., 2009; Nishitani et al., 2002). It is currently suggested that the potent hypoglycemic effect of amino acids is mediated by phosphoinositide 3-kinase (PI3K) and atypical isoforms of protein kinase C (aPKC) (Doi et al., 2003; Morifuji et al., 2009; Nishitani et al., 2002). Therefore, it may be possible to develop an amino acid supplement as a potent non-pharmaceutical approach to improve glucose tolerance.

Our laboratory recently found that gavaging Sprague-Dawley rats with an amino acid mixture, composed of isoleucine, leucine, cystine, methionine, and valine, improved the blood glucose response during an oral glucose challenge without an increase in the plasma insulin response (Bernard et al., 2011). The blood glucose-lowering effect of the amino acid mixture was due to an increase in skeletal muscle glucose uptake. These results suggest that this amino acid supplement acutely improves muscle insulin sensitivity and blood glucose homeostasis in rats.

Although our previous results support the hypoglycemic effect of an amino acid supplement in rats, the effect of this supplement on blood glucose homeostasis has not been evaluated in humans. According to data from the National Center for Health Statistics 2010 (2011), it is estimated that two-thirds of the adult population and a growing number of children in the United States are overweight or obese. Weight is considered the single strongest predictor of type 2 diabetes (Laaksonen et al., 2009). Therefore, the objective of the present investigation was to study whether the ingestion of an amino acid mixture, composed of isoleucine and 4 additional amino acids, could improve the blood glucose clearance of healthy overweight/obese men and women during an oral glucose tolerance test. We hypothesized that the amino acid mixture would significantly reduce the blood glucose response to a glucose challenge in healthy overweight/obese subjects, and this blood glucose-lowering effect would be insulin independent.

EXPERIMENTAL PROCEDURES

This study was registered as a clinical trial with http://www.ClinicalTrials.gov/ NCT00974831.

Subjects. Twenty-two healthy overweight/obese men and women (3 men and 19 women) between 20 and 45 years old volunteered for the study. Subject characteristics are presented in Table 3.1. The subjects habitually engaged in recreational exercise less than 3 hrs per week and were considered sedentary. They were not engaged in any exercise programs over the course of the experiment. A body mass index (BMI) of more than 25 kg/m^2 was required for all subjects. A waist circumference of at least 101 cm was required for all male subjects and 88 cm for female subjects. All subjects' fasting blood glucose levels were between 4.16 and 6.99 mmol/L (75 and 126 mg/dL), and their blood pressures were less than 140/90 mm Hg. The subjects were first screened via phone to determine if they met the requirements of age, exercise level, and health status, and were not on a low carbohydrate diet before they came for the screening visit. During the screening visit, the experimental procedures and potential risks of the study were fully explained to the subjects, and all subjects signed the *Informed Consent to Participate in Research* and filled out the *Participation Health Research Screening Form*. The experimental protocol was approved by The University of Texas at Austin Institutional Review Board.

Physical Activity and Nutritional Controls. Subjects were asked to keep their habitual exercise pattern and normal dietary intake as constant as possible over the course of the experiment. They were instructed to consume their last meal at least 14 hrs prior to their trial appointment, during which they were allowed to consume only water and an 8 oz can of Ensure (Abbott Nutrition, Columbus, OH). The 8 oz can of Ensure was consumed 12 hrs prior to each of the trials. All subjects were required to bring a 3-day food record for analysis of their daily average intake of carbohydrate and they were advised to eat the same diet 3 days prior to each trial. The subjects were also refrained from exercise for 24 hrs before each of the trials. On reporting to the laboratory, the three-day food record was analyzed to ensure that subjects had consumed a minimum of 150 g of carbohydrate per day. All subjects were in compliance with these instructions.

Experimental Design. Upon reporting to the laboratory, the subjects were weighed and a finger prick glucose test was performed to ensure the blood glucose level of each subject was between 4.16 mmol/L and 6.99 mmol/L. After the subject sat quietly for at least 5 min, a catheter was inserted into a forearm vein, fitted with a three-way

stopcock with a catheter-extension, and taped in place. Each subject completed two glucose tolerance tests after consuming two different test beverages using a double-blinded, random ordered experimental design. Tests were separated by at least 1 week but not more than 2 weeks. All beverages were provided by Abbott Nutrition (Columbus, OH). The CHO/AA beverage consisted of an amino acid mixture in a 355 ml orange-flavored solution. The amino acid mixture consisted of 0.088 g cystine 2HCl, 0.043 g methionine, 0.086 g valine, 12.094 g isoleucine and 0.084 g leucine per 355 ml solution. The CHO beverage consisted of a 355 ml orange-flavored placebo drink. Before each trial, both beverages were mixed with a 296 ml orange-flavored drink containing 100 g dextrose (SUN-DEX, Fisher Healthcare, Houston, TX) to make a drink totaling 651 ml. The experimental drinks were provided to subjects every 5 min in 207 ml, 237 ml and 207 ml increments. All subjects were instructed to finish the drinks within a total of 15 min. Subjects rested quietly in a room with dimmed lighting during each trial. Venous blood samples (7 ml) were drawn 10 min before the start of ingesting the drinks. Once the drinks were ingested, subsequent blood samples (7 ml) were taken at 15, 30, 60, 120 and 180 min.

Blood Analysis. Each blood sample was transferred into two 12 x 75 mm polypropylene culture test tubes cooled on ice, containing ethylenediaminetetraacetic acid solution (EDTA, 24 mg/ml, pH 7.4). From all blood samples, 0.3 ml of the anticoagulated blood was transferred to tubes containing 0.6 ml 10% perchloric acid (PCA). All tubes were centrifuged for 10 min at 3,000 rpm at 4℃ in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific Inc. Waltham, MA). Following centrifugation, the PCA extracts were transferred to 12 x 75 test tubes and stored for later analysis of blood lactate. The plasma was transferred to five new 12 x 75 test tubes. Three tubes received 0.6 ml plasma each and were stored for later analysis of insulin, c-peptide, and glucagon. A protease inhibitor, Trasylol (aprotinin), was added to the glucagon test tubes to prevent proteolysis. A fourth test tube received 0.5 ml plasma and was stored for later analysis of glucose, free fatty acids (FFA), and triglycerides (TG). The remaining plasma was placed in a fifth test tube and saved as a backup. All test tubes were immediately stored at -80℃. Each subject's blood samples were analyzed in duplicate and concurrently after completion of all trials.

Plasma glucose was determined using a colorimetric method, which employs glucose oxidase (GOD) and a modified Trinder color reaction (Trinder, 1969). The absorbance of the reaction solution was measured using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at a wavelength of 500 nm. The intensity of the color in the reaction solution was proportional to the concentration of glucose in the blood sample. Duplicate samples were measured and the intra-assay coefficient of variance was 2.20%.

Plasma insulin, c-peptide, and glucagon were measured using commercially available ^{125}I radioimmunoassay (RIA) kits (MP biomedicals, LLC. Solon, OH). The radioactivity of antibody-bound ^{125}I-labeled hormone was counted in a Wallac 1470 Wizard™ automatic gamma counter (Perkin Elmer life Sciences, Turku, Finland). The concentrations of plasma hormones were calculated based on prepared standards. Duplicate samples were measured and the intra-assay coefficient of variances for insulin, c-peptide, and glucagon assays were 4.46%, 4.20%, and 8.10%, respectively.

FFA was measured using a modified colorimetric method stemming from those devised by Ayers (Ayers, 1956) and Iwayama (Iwayama, 1959). The absorbance of the reaction solution was measured using a Beckman DU 640 spectrophotometer at a wavelength of 436 nm. Duplicate samples were measured and the intra-assay coefficient of variance was 5.52%.

Lactate was determined spectrophotometrically as described by Hohorst (Hohorst, 1965). The reaction can be monitored by the changes in the fluorescence of NADH, which was measured using a Beckman DU 640 spectrophotometer at a wavelength of 340 nm. Duplicate samples were measured and the intra-assay coefficient of variance was 1.31%.

TG was measured using triglyceride GPO reagent (Cliniqa corporation, San Marcos, CA). The absorbance of the reaction solution was measured using a Cary 50 MPR microplate reader (Varian Australia Pty Ltd, Victoria, Australia) at a wavelength of 520 nm. The absorbance of quinoneimine dye in the reaction solution was proportional to the concentration of TG in the blood sample. Duplicate samples were measured and the intra-assay coefficient of variance was 10.07%.

Statistics. Statistical analyses were performed by using SPSS software (SPSS Inc, Chicago, IL). The data were analyzed using a two-way ANOVA (treatment x time) with repeated measures. When a significant F-ratio was obtained, a Bonferroni post hoc test was made to identify significant differences between means. Paired t-tests were performed to determine if significant differences in the area under the curve (AUC) for glucose, insulin, c-peptide, and glucagon occurred between treatments. A power analysis was conducted using MANOVA per D'Amico (D'Amico et al., 2001) using a correlation matrix obtained from a previous rat study data (power = 0.88). Effect sizes were derived from correlation matrices from a previous rat study data. A MANOVA per D'Amico (D'Amico et al., 2001) was then run to project sample size using repeated measures. Sensitivity analysis was performed to determine minimum and maximum sample sizes (max. sample size = 14, min. sample size = 10). A sample of 22 was chosen as a conservative estimate to maintain power over 0.9. Statistical significance was set at $p<0.05$. All data are displayed as mean ± standard error (SE).

RESULTS

The mean fasting blood glucose values were not significantly different between the two treatments (CHO/AA 4.18±0.07 mmol/L, CHO 4.16±0.06 mmol/L) (Fig. 3.1A). However, there was a significant treatment effect with the blood glucose response for the CHO/AA treatment significantly lower than that of the CHO treatment. This result was supported by the finding that the glucose AUC for the CHO/AA treatment was significantly lower than that for the CHO treatment (Fig. 3.1B). Statistical analysis also identified a treatment by time interaction with blood glucose during the CHO/AA treatment lower than the CHO treatment at 30, 60, 120, and 180 min after supplement ingestion. It was further noted that blood glucose returned to the fasting level after 120 min during the CHO/AA treatment, but during the CHO treatment this required 180 min.

The mean fasting plasma insulin values were not significantly different between the two treatments (CHO/AA 161.34±13.66 pmol/L, CHO 163.80 ± 14.53 pmol/L) (Fig. 3.2A). Furthermore, there was no significant difference between the CHO and CHO/AA treatments, nor was there a significant difference in the insulin AUC between the two treatments (Fig. 3.2B). After ingestion of the beverage, blood insulin increased

immediately and remained higher than the fasting value for the next 180 min after both treatments, and there were no significant differences in plasma insulin concentration between the two treatments at any time point.

Similar to the plasma insulin responses, plasma c-peptide concentrations did not differ between the treatments at any time point (Fig. 3.3A). There was also no significant difference in the c-peptide AUC between the two treatments (Fig. 3.3B). After ingestion of either treatment, plasma c-peptide increased and remained higher than the fasting value for the next 180 min. The maximal c-peptide concentration was reached at 60 min in both treatments.

The mean fasting plasma glucagon values were not significantly different between the two treatments (CHO/AA 63.85±5.91 ng/L, CHO 67.70±6.52 ng/L) (Fig. 3.4A). There was, however, a significant treatment effect found between the CHO and CHO/AA treatments. There was also a significant difference in the glucagon AUC between the two treatments (Fig. 3.4B). As expected, the plasma glucagon concentration significantly decreased at 30 min during the CHO treatment and remained below the fasting value for the next 180 min. During the CHO/AA treatment, the plasma glucagon concentration fluctuated around the fasting value during the OGTT, but never became significantly lower than the fasting value. The plasma glucagon concentration was significantly higher in CHO/AA than CHO at 60 min post treatment ($p<.05$).

The mean fasting plasma FFA concentration was significantly lower in CHO/AA treatment as compared to CHO treatment (CHO/AA 0.224±0.014 mmol/L, CHO 0.257±0.014 mmol/L, $p<.05$) (Fig. 3.5A). There was no significant difference between the CHO and CHO/AA treatments. However, there was a significant treatment by time interaction with plasma FFA during the CHO/AA treatment lower than the CHO treatment at 15 min after supplement ingestion. There were no significant differences in plasma FFA concentration between the two treatments at any other time point post beverage ingestion. Plasma FFA concentration significantly decreased at 30 min and reached a plateau at 120 min during both treatments.

No treatment effect was found for plasma TG (Fig. 3.5B) or blood lactate (Fig. 3.6). Moreover, there were no significant differences in plasma TG or blood lactate concentrations at any time point between the two treatments.

DISCUSSION

In the present study, we investigated the acute effect of an amino acid mixture on glucose tolerance in healthy overweight/obese adults. We found that the amino acid mixture, composed of isoleucine, cystine, methionine, valine, and leucine, can improve the glucose response to an oral glucose challenge. In addition, we found that the blood glucose response was significantly reduced within 30 min of ingesting the glucose/amino acid mixture (Fig. 3.1A). Moreover, blood glucose returned to the fasting level more rapidly during the CHO/AA treatment than the CHO treatment. These results are consistent with our previous study, which shows that amino acid mixture can reduce blood glucose responses to an oral glucose challenge in Sprague-Dawley rats (Bernard et al., 2011). Ikehara et al. (Ikehara et al., 2008) also reported a hypoglycemic effect of isoleucine, the primary amino acid in our mixture. In their study, mice fasted for 18h were given isoleucine or distilled water orally. During the following oral glucose tolerance test, the blood glucose level was significantly reduced by isoleucine administration in normal mice, as well as in diet-induced glucose-intolerant mice and db/db mice, a model of severe type 2 diabetes.

Some studies ascribe the hypoglycemic effects of amino acids to an increased insulin response (Kalogeropoulou et al., 2008). It is known that protein and certain amino acids, such as leucine, isoleucine, arginine, and methionine, are insulin secretagogues (Boden and Tappy, 1990; Bolea et al., 1997; Broca et al., 1999; Broca et al., 2000; Fajans et al., 1967; Milner, 1970; Sener and Malaisse, 1981; Sener et al., 1981; Spiller et al., 1987). Protein and amino acids have been reported to increase the blood insulin response and reduce the blood glucose response when ingested with carbohydrate (Kalogeropoulou et al., 2008; Spiller et al., 1987; van Loon et al., 2000; Zawadzki et al., 1992). Kalogeropoulou et al. (Kalogeropoulou et al., 2008) reported that 1 mmol/kg lean body mass leucine (a mean of 7 g) provided with 25 g glucose attenuated the blood glucose response and strongly stimulated additional insulin secretion in healthy subjects, compared with a 25 g glucose only treatment. Moreover, Nuttall et al. (Nuttall, 2008) reported that 1 mmol/kg lean body mass isoleucine (a mean of 7.4 g) reduced the blood glucose response without a significant rise in plasma insulin concentration. Several animal studies have also suggested the insulin-independent hypoglycemic effect of amino

acids *in vivo* (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007; Ikehara et al., 2008) and *in vitro* (Doi et al., 2003; Nishitani et al., 2002). Our results are consistent with an insulin-independent effect of amino acids on control of blood glucose. Although CHO/AA significantly reduced the blood glucose response, the plasma insulin responses were similar between CHO/AA and CHO treatments. This was further confirmed by the similar c-peptide responses in both treatments. Therefore, the improved glucose tolerance by the amino acid mixture in the present study was not likely due to an increased plasma insulin response.

Aside from an increased insulin response, there are several other possibilities that might account for the reduced glucose response to the glucose challenge during the CHO/AA treatment. One is that the amino acid mixture slowed gastric emptying, resulting in a smaller blood glucose response. Dietary protein before or with a meal generally slows gastric emptying (Bowen et al., 2006) and produces greater satiety (Latner and Schwartz, 1999; Ma et al., 2009; Poppitt et al., 1998; Porrini et al., 1995). However, the similar insulin and c-peptide responses for both treatments in the present study suggest that the appearance rate of glucose from the gastrointestinal system to the circulation should be similar. Furthermore, it is unlikely the small amount of amino acids ingested during the OGTT would have the same effect on gastric emptying as dietary protein.

Another possibility is that the amino acid mixture suppressed hepatic glucose output. Doi et al. (Doi et al., 2007) reported that under insulin-free conditions, isoleucine significantly inhibited hepatic gluconeogenesis when alanine was used as a glucogenic substrate in isolated hepatocytes. This was accompanied by a reduction in mRNA levels for phosphoenolpyruvate carboxykinase (PEPCK), a key gluconeogenic enzyme, and a decrease in PEPCK activity. A reduction in mRNA levels for glucose-6-phosphatase (G6Pase) and a decreased activity of G6Pase were also found in both isoleucine-incubated hepatocytes and isoleucine-administered rats. These findings suggest that the hypoglycemic effect of amino acids could be due, in part, to a reduced hepatic glucose output subsequent to inhibition of gluconeogenesis. However, with plasma insulin levels reaching 1200 pmol/L in both the CHO/AA and CHO treatments, it is likely that

gluconeogenesis was completely suppressed, and any effect amino acids could have on hepatic glucose output was negated (Ader and Bergman, 1990; Sindelar et al., 1997).

A third possibility for the hypoglycemic effect of the amino acid mixture in the present study was an acceleration in peripheral tissue glucose uptake. Several *in vivo* animal studies have reported that certain amino acids increase skeletal muscle glucose uptake (Bernard et al., 2011; Doi et al., 2005; Doi et al., 2007; Kleinert et al., 2011). For example, Doi et al. (Doi et al., 2007) found that oral administration of isoleucine (0.45g/kg body weight) with a bolus intravenous administration of 2-deoxyglucose significantly decreased the plasma glucose level by 20% and enhanced skeletal muscle glucose uptake by 71% in rats without a significant rise in plasma insulin. In contrast, no significant changes in glucose uptake were identified in the liver or adipose tissue. Likewise, Bernard et al. (Bernard et al., 2011) reported that our amino acid mixture increased muscle glucose uptake in both fast-twitch red and white muscles with no difference in insulin response among treatments. These results suggest that the hypoglycemic effect of our amino acid mixture is due to an increased glucose uptake in skeletal muscle.

The mechanism by which amino acids increase muscle glucose uptake is not immediately clear. The possible underlying signaling pathway for amino acid-stimulated glucose uptake in skeletal muscle was investigated in several *in vitro* studies (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007; Kleinert et al., 2011; Morifuji et al., 2009; Nishitani et al., 2002). Doi et al. (Doi et al., 2003) found that isoleucine increased glucose uptake of C2C12 myotubes in an insulin-independent manner. Nishitani et al. (Nishitani et al., 2002) also found that 2 mM leucine significantly increased 2-deoxyglucose uptake in isolated rat soleus muscle under insulin-free conditions. It has been reported that PI3K was transiently activated in myotubes by leucine (Peyrollier et al., 2000), which is a key enzyme related to the translocation of GLUT4 to the plasma membrane and glucose uptake in skeletal muscle. However, the phosphorylation of Akt/PKB, which is an intermediate in the insulin signaling pathway and lies downstream of PI3K, was unchanged in myotubes by incubation with leucine or isoleucine (Atherton et al. 2010; Kimball et al., 1999; Peyrollier et al., 2000). Analysis of the intracellular signaling pathway via enzyme inhibitors found that the PI3K inhibitor LY294002 and aPKC

inhibitor GF 109203X independently suppressed glucose uptake stimulated by isoleucine or leucine (Doi et al., 2003; Nishitani et al., 2002). Inhibition of the mammalian target of rapamycin (mTOR) with rapamycin, however, had no effect on amino acid-stimulated glucose transport (Doi et al., 2003; Nishitani et al., 2002). A recent study from our laboratory found that our amino acid mixture significantly increased glucose uptake in isolated epitrochlearis muscle in the absence or presence of a submaximal and maximal insulin concentration (Kleinert et al., 2011). Western blotting analysis revealed that the amino acid mixture increased phosphorylation of Akt substrate of 160 kDa (AS160) under these conditions. AS160 is one potential candidate of downstream targets of Akt/PKB and has been suggested to regulate GLUT-4 translocation in response to either insulin or contractile activity (Bruss et al., 2005; Cheng et al., 2005; Eguez et al., 2005). However, our amino acid mixture had no effect on Akt/PKB or mTOR phosphorylation. These results were confirmed with an *in vivo* study from our laboratory. Intubation of our amino acid mixture significantly elevated muscle glucose uptake in Sprague-Dawley rat independently of insulin (Bernard et al., 2011). The increased muscle glucose uptake was accompanied by an increased phosphorylation of AS160 without altering Akt/PKB activation. These findings suggest that certain amino acids may enhance muscle glucose transport through an as yet undefined intracellular signaling pathway.

Although previous studies and our findings suggest improved glucose tolerance by protein and amino acid supplementation, others have reported that amino acids inhibit muscle glucose uptake rather than improve glucose tolerance (Abumrad et al., 1982; Baum et al., 2005; Flakoll et al., 1992; Patti et al., 1998; Pisters et al., 1991; Tessari et al., 1985). It is proposed that amino acids activate mTOR/p70S6K signaling, which mediates the phosphorylation of insulin receptor substrate 1 (IRS-1) and suppresses IRS-1-dependent PI3K/Akt signaling for glucose transport (Iwanaka et al., 2010; Patti et al., 1998; Tremblay and Marette, 2001; Tzatsos and Kandror, 2006). Of all the essential amino acids, leucine has been suggested as the most potent and unique in stimulating mTOR/p70S6K signaling (Anthony et al., 2000; Atherton et al., 2010; Blomstrand et al., 2006; Patti et al., 1998; Tzatsos and Kandror, 2006). However, the amino acid mixture investigated in the present study was predominately composed of isoleucine, which has been shown to have no effect on mTOR and p70S6K (Atherton et al., 2010; Tremblay

and Marette, 2001). Therefore, the different compositions of amino acids utilized may be responsible for the observed conflicting effects of amino acids on blood glucose regulation.

Other possibilities for the conflicting effects of amino acids on blood glucose may be the different amounts, exposure time, and methods of administering the amino acids. It was found that *in vitro* muscle glucose uptake was optimal with 2 mM leucine but decreased with 4 mM leucine under insulin-free conditions (Nishitani et al., 2002). Therefore, there may be an optimal concentration range of amino acids, which can stimulate muscle glucose uptake. Below or above this optimal range may limit the glucose lowering effect of amino acids. Moreover, Nishitani et al. (Nishitani et al., 2002) found that muscle glucose uptake *in vitro* increased when the muscle was incubated with 2 mM leucine for 15-45 min but not with 60 min incubation. Therefore, over exposure to high concentrations of amino acids could result in insulin resistance and limit blood glucose uptake. In addition, Tremblay et al. (Tremblay et al., 2005b) found that amino acid infusion rapidly increased the plasma amino acid concentration and decreased the whole body rate of glucose disappearance in response to peripheral hyperinsulinemia. The resulting hyperaminoacidaemia may suppress glucose transport via the overactivation of mTOR/p70S6K, which can suppress the IRS-1-dependent PI3K/Akt signaling cascade. Therefore, hyperaminoacidaemic infusion may also be partially contributing to the conflicting effects of amino acids on blood glucose regulation.

The present study found that the plasma glucagon concentration was significantly decreased after 30 min of CHO ingestion. However, when amino acid mixture was ingested with glucose, the plasma glucagon concentration fluctuated around the fasting value for the entire 3 hrs and was significantly higher than CHO at 60 min. The relative higher glucagon concentration in CHO/AA, as compared to the CHO treatment, may be due to the reduced glucose concentration by the amino acid mixture. The blood glucose concentration was significantly lower in CHO/AA compared with the CHO treatment (CHO/AA 4.71 mmol/L, CHO 5.95 mmol/L) at 60 min post supplement, which corresponded with the significantly higher plasma concentration of glucagon at 60 min in CHO/AA.

Another reason for the higher glucagon level in CHO/AA may be the amino acid mixture itself. It has been reported that protein and amino acids stimulate an increase in circulating glucagon concentration (Unger and Orci, 1976; Westphal et al., 1990). Nuttall et al. (Nuttall, 2008) reported a small, transient increase in glucagon in subjects given isoleucine at a concentration of 1 mmol/kg lean body mass. Kalogeropoulou et al. (Kalogeropoulou et al., 2008) also recently reported an increase in glucagon concentration with orally administrated leucine. Glucagon is known to stimulate the liver to release glucose and raise blood glucose levels (Samols et al., 1966; Vranic et al., 1976). However, in the present study, the blood glucose concentration was significantly lower in CHO/AA compared with the CHO treatment, even though the plasma glucagon level was significantly higher in CHO/AA. These results, therefore, further support the hypoglycemic effect of the amino acid mixture.

Although the mean fasting plasma FFA concentration was significantly lower in CHO/AA trial compared to CHO, there were no significant differences in plasma FFA concentration between the two treatments at any time point except at 15 min post beverage ingestion. This difference in FFA concentration at 15 min between the two treatments was likely caused by the initial difference in mean fasting FFA concentrations. Likewise, there were no significant differences in plasma TG at any time between the two treatments. Therefore, the amino acid mixture appeared to have little effect on plasma FFA or TG metabolism during the oral glucose challenge.

It is well known that blood lactate concentration increases after an oral glucose load (Bratusch-Marrain et al., 1980; Doar et al., 1970; Prando et al., 1988). The rise in blood lactate is caused by an inhibition of splanchnic lactate uptake and an increase in hepatic and muscle lactate production. However, the contribution to a rise in blood lactate is mainly a result of enhanced glycolysis in muscle, rather than a reduced lactate uptake by the liver (Bratusch-Marrain et al., 1980; Prando et al., 1988). In the present study, as expected, the plasma lactate concentration increased and peaked at 60 min after both supplements. There was no significant difference in blood lactate between the two treatments at any time point. This may suggest that an increased glucose clearance via the amino acid supplement is directed more to glucose storage than glycolysis, if in fact the

lower blood glucose level during the CHO/AA treatment was due to an increase in glucose uptake by skeletal muscle.

In summary, an amino acid mixture, composed of isoleucine and 4 additional amino acids, lowered the glucose response to an OGTT in healthy overweight/obese subjects. Insulin was not likely responsible for the smaller increase in blood glucose concentration during the CHO/AA treatment since the plasma insulin and c-peptide responses were similar in both treatments. There was a small increase in plasma glucagon during the CHO/AA treatment, as compared with the CHO treatment, which was most likely due to the lower blood glucose response during the CHO/AA treatment. There were no differences in plasma FFA, TG, or blood lactate between the two treatments at any time point. This study suggests that a mixture of amino acids may be effective in improving the control of blood glucose in individuals at risk of developing type 2 diabetes. Further research is needed to determine if type 2 diabetics will respond favorably to amino acid therapy.

	Male	Female
Number	3	19
Age (yr)	25 ± 1.53	31.79 ± 1.75
Weight (kg)	103.31 ± 6.77	91.02 ± 2.65
Height (cm)	180.87 ± 0.62	161.19 ± 2.03
BMI (kg/m^2)	31.53 ± 2.15	35.04 ± 1.16
Waist circumference (cm)	105.67 ± 1.20	105.58 ± 2.27
Screen Fasting blood glucose (mmol/L)	4.92 ± 0.46	5.52 ± 0.13

Values are expressed as means + SE. BMI, body mass index. Fasting blood glucose was measured during screening visit.

Table 3.1 Characteristics of subjects

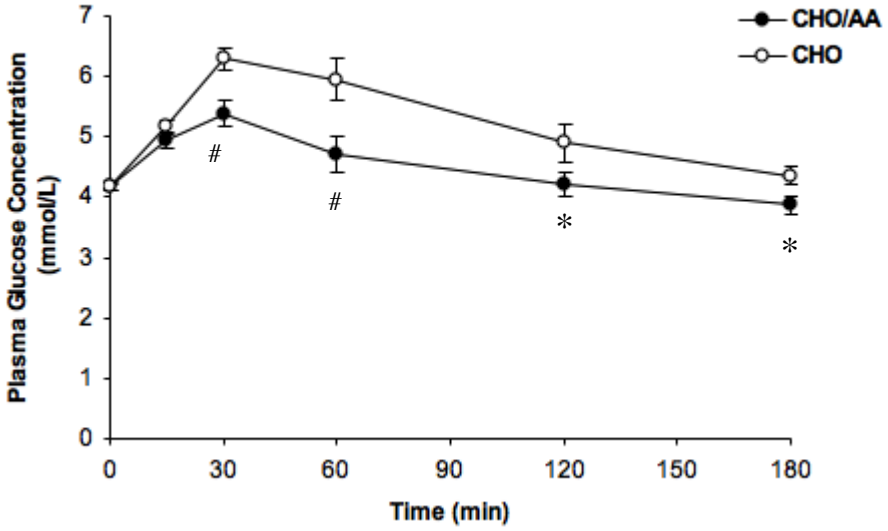


FIGURE 3.1A. BLOOD GLUCOSE DURING OGTT. 22 subjects ingested 100 g dextrose (CHO) or 100 g dextrose plus a 5 amino acid mixture (CHO/AA). Blood was taken before the ingestion and 15, 30, 60, 120 and 180 min after. Values are means ± SE. *, $p <$ 0.05 CHO/AA vs. CHO. #, $p<0.001$ CHO/AA vs. CHO.

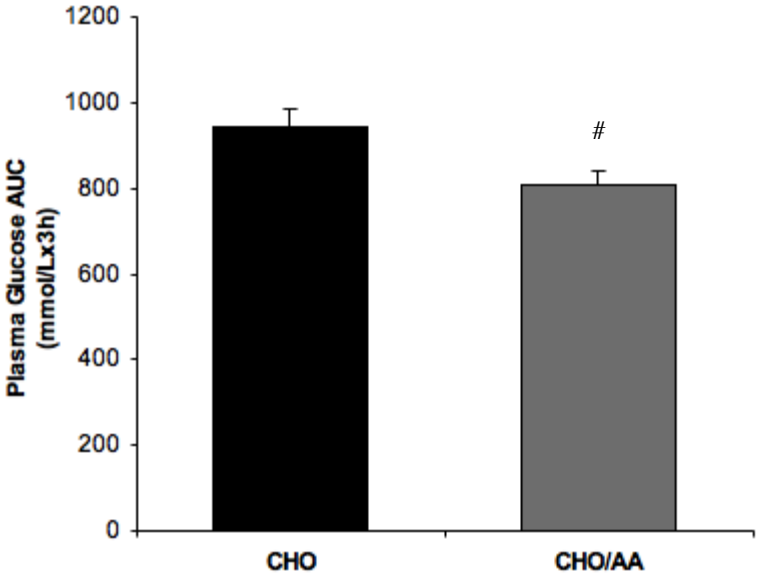


FIGURE 3.1B. BLOOD GLUCOSE AREA UNDER THE CURVE (AUC) DURING OGTT. AUC was calculated with baseline (pre). Values are means ± SE. #, $p < 0.001$ CHO/AA vs CHO.

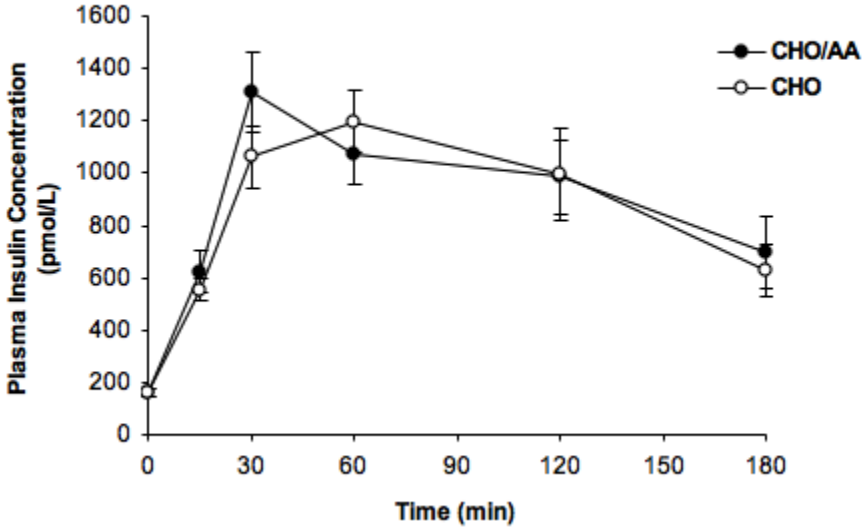


FIGURE 3.2A. PLASMA INSULIN DURING OGTT. 22 subjects ingested 100 g dextrose (CHO) or 100 g dextrose plus a 5 amino acid mixture (CHO/AA). Blood was taken before the ingestion and 15, 30, 60, 120 and 180 min after. Plasma insulin was determined by radioimmunoassay. Values are means ± SE.

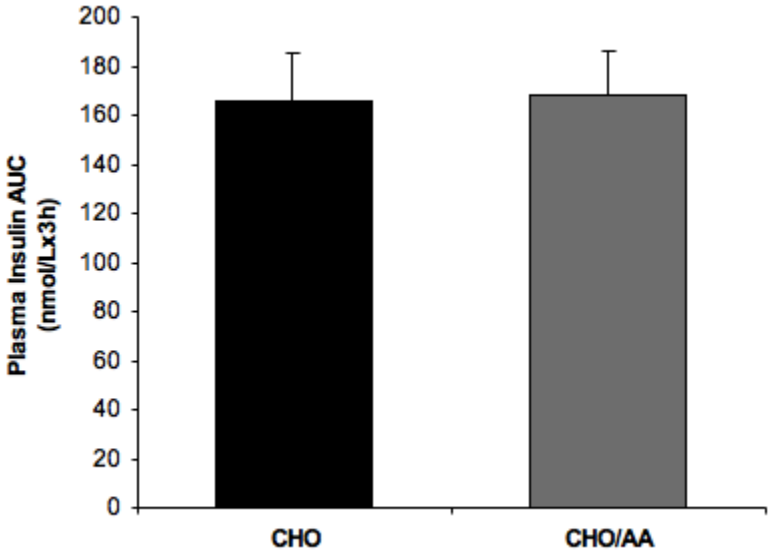


FIGURE 3.2B. PLASMA INSULIN AREA UNDER THE CURVE (AUC) DURING OGTT. AUC was calculated with baseline (pre). Values are means ± SE.

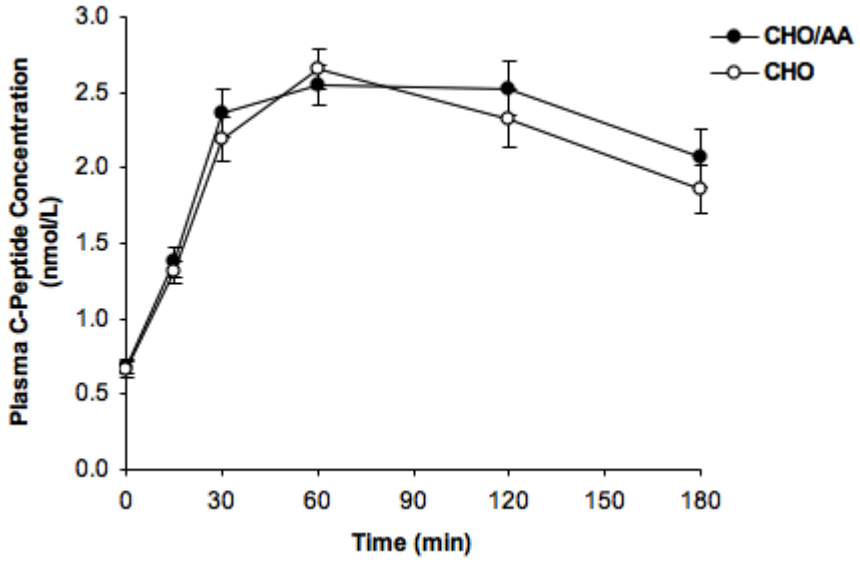


FIGURE 3.3A. PLASMA C-PEPTIDE DURING OGTT. 22 subjects ingested 100 g dextrose (CHO) or 100 g dextrose plus a 5 amino acid mixture (CHO/AA). Blood was taken before the ingestion and 15, 30, 60, 120 and 180 min after. Plasma C-peptide was determined by radioimmunoassay. Values are means ± SE.

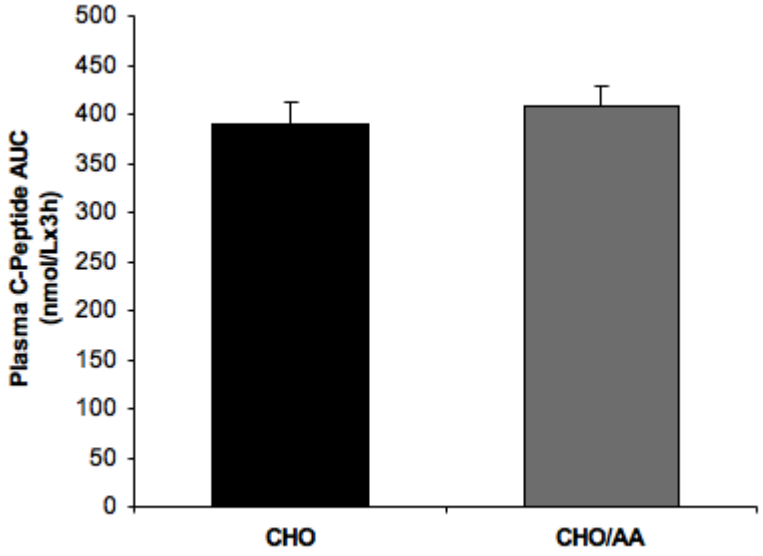


FIGURE 3.3B. PLASMA C-PEPTIDE AREA UNDER THE CURVE (AUC) DURING OGTT. AUC was calculated with baseline (pre). Values are means ± SE.

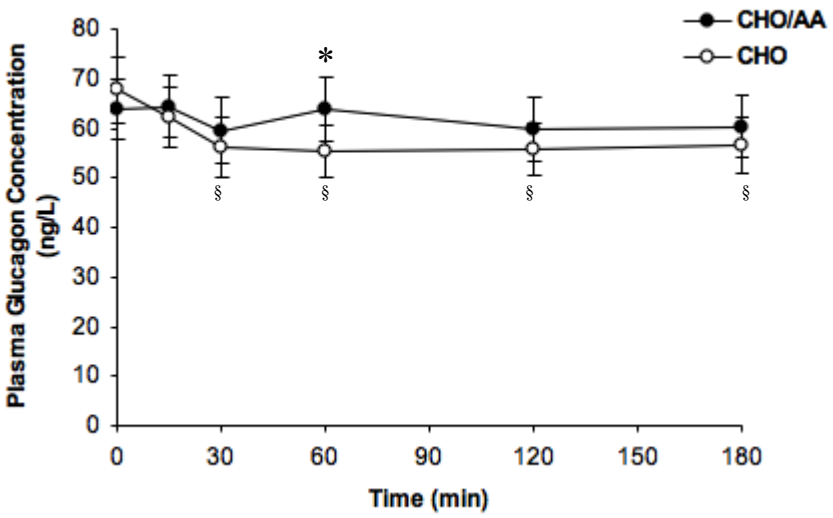


FIGURE 3.4A. PLASMA GLUCAGON DURING OGTT. 22 subjects ingested 100 g dextrose (CHO) or 100 g dextrose plus a 5 amino acid mixture (CHO/AA). Blood was taken before the ingestion and 15, 30, 60, 120 and 180 min after. Plasma glucagon was determined by radioimmunoassay. Values are means ± SE. *, $p < 0.05$ CHO/AA vs. CHO. §, $p<0.05$ vs baseline (time 0).

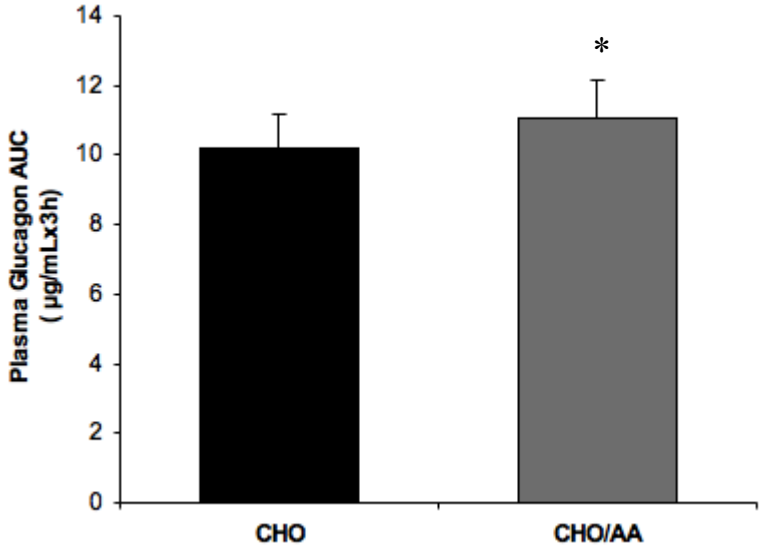


FIGURE 3.4B. PLASMA GLUCAGON AREA UNDER THE CURVE (AUC) DURING OGTT. AUC was calculated with baseline (pre). Values are means ± SE. *, $p < 0.05$ CHO/AA vs. CHO.

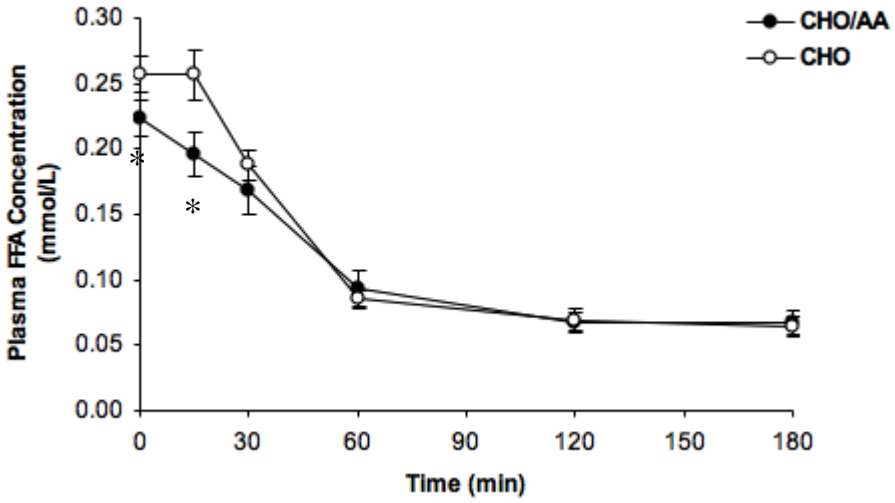


FIGURE 3.5A. PLASMA FFA DURING OGTT. 22 subjects ingested 100 g dextrose (CHO) or 100 g dextrose plus a 5 amino acid mixture (CHO/AA). Blood was taken before the ingestion and 15, 30, 60, 120 and 180 min after. Values are means ± SE. *, $p < 0.05$ CHO/AA vs. CHO.

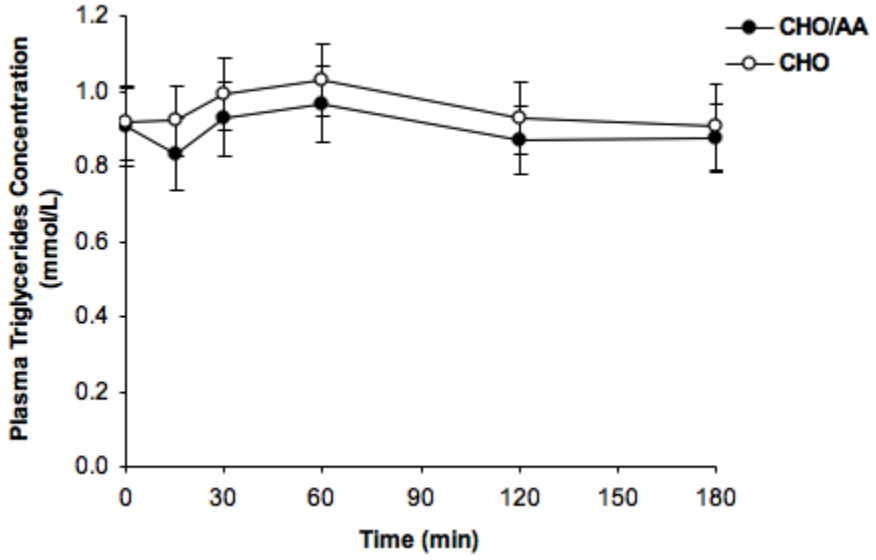


FIGURE 3.5B. PLASMA TRIGLYCERIDES DURING OGTT. 22 subjects ingested 100 g dextrose (CHO) or 100 g dextrose plus a 5 amino acid mixture (CHO/AA). Blood was taken before the ingestion and 15, 30, 60, 120 and 180 min after. Values are means ± SE.

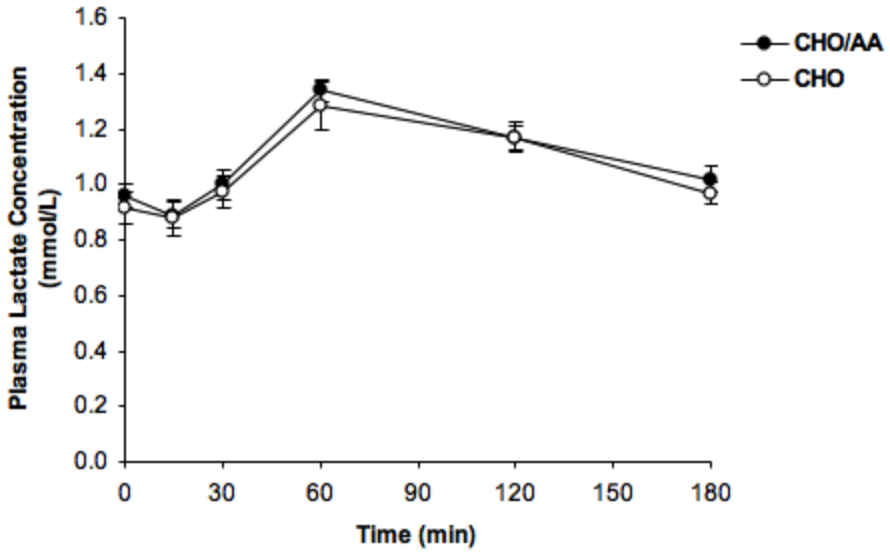


FIGURE 3.6. PLASMA LACTATE DURING OGTT. 22 subjects ingested 100 g dextrose (CHO) or 100 g dextrose plus a 5 amino acid mixture (CHO/AA). Blood was taken before the ingestion and 15, 30, 60, 120 and 180 min after. Values are means $\pm$ SE

Chapter IV: The Effect of an Amino Acid Beverage on Glucose Response, Glycogen Replenishment, Muscle Damage, and Anaerobic Performance after Strenuous Exercise

ABSTRACT

In the present study, we tested whether an amino acid mixture, composed of isoleucine and 4 other amino acids, would affect blood glucose and muscle glycogen replenishment when a carbohydrate supplement was provided after strenuous exercise. The effects of the amino acid mixture on muscle damage and subsequent anaerobic performance were also evaluated. After an intense glycogen-depleting cycling bout, subjects received a CHO/HAA (1.2g/kg bw CHO, 13 g amino acid mixture), CHO/LAA (1.2g/kg bw CHO, 6 g amino acid mixture), or CHO (1.2g/kg bw CHO) supplement immediately after exercise and 2 hr postexercise. Muscle biopsies were performed immediately post exercise and 4 hr postexercise. After the second biopsy, a Wingate Anaerobic Test (WAnT) was performed to evaluate anaerobic performance. The CHO/HAA and CHO/LAA treatments significantly decreased glucose response compared with CHO. The CHO/HAA caused a significantly higher insulin response compared with CHO treatment. Glycogen storage rate was significantly lower in the CHO/HAA compared with CHO while it did not differ significantly between the CHO/LAA and CHO treatments (CHO/HAA 15.4 ± 2.0 μmol/g wet muscle•4h^{-1}, CHO/LAA 18.1 ± 2.0 μmol/g wet muscle•4h^{-1}, CHO 21.5 ± 1.4 μmol/g wet muscle•4h^{-1}). There were no significant differences in the blood lactate, creatine kinase, or myoglobin responses among treatments. The CHO/HAA had a greater effect on phosphorylation of mTOR and Akt/PKB compared with CHO treatment. No significant differences in AS160 or GS phosphorylation were identified between treatments. The results suggest that the small dosage of amino acid mixture is effective in controlling blood glucose. The higher dosage of amino acid mixture also may stimulate insulin secretion and activate insulin-induced signaling.

INTRODUCTION

Several studies have reported that leucine and/or isoleucine can stimulate glucose transport independently of insulin, possibly via an mTOR-independent PI3K/aPKC pathway (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007; Nishitani et al., 2002). Glucose uptake by skeletal muscle may be the major mechanism by which leucine and/or isoleucine lowers blood glucose levels (Bernard et al., 2011; Doi et al., 2005; Doi et al., 2007; Kleinert et al., 2011). The storage form of glucose in the muscles, muscle glycogen, is an important fuel source for moderate to high intensity exercise. The faster the muscle glycogen can be replenished, the greater ability to achieve optimal performance in subsequent exercise. Therefore, faster clearance of blood glucose by skeletal muscle caused by amino acids may enhance muscle glycogen synthesis and be beneficial for subsequent exercise.

Recent studies have shown that daily supplementation or acute ingestion of branched-chain amino acids (BCAAs) may prevent muscle damage that occurs resulting from prolonged, intense endurance exercise or strength training (Coombes and McNaughton, 2000; Greer et al., 2007; Jackman et al., 2010; Shimomura et al., 2010; Skillen et al., 2008). Muscle damage could affect force production and result in a reduction in the subsequent anaerobic exercise performance. Based on the fact that amino acids have been found to prevent exercise-induced muscle damage, it is reasonable to hypothesize that supplementing post-exercise with carbohydrate plus amino acids will lead to a faster post-exercise recovery and improve subsequent anaerobic exercise performance.

Our laboratory recently found that gavaging normal Sprague-Dawley rats with an amino acid mixture, composed of isoleucine, leucine, cystine, methionine, and valine, improved the blood glucose response during an oral glucose challenge without an increase in the plasma insulin response (Bernard et al., 2011). The blood glucose-lowering effect of the amino acid mixture was due to an increase in skeletal muscle glucose uptake. Correspondingly, it was also found that the amino acid mixture enhanced glucose uptake in rat epitrochlearis muscle in the absence of insulin, and augmented insulin-stimulated glucose uptake in an additive manner when the muscle was treated with physiological and maximally-stimulating concentrations of insulin (Kleinert et al.,

2011). The hypoglycemic effect of the amino acid mixture appears to be mediated by a molecular pathway that is independent from the insulin signaling cascade. Moreover, we have recently found that the similar amino acid mixture lowered the blood glucose response and improved glucose tolerance during an OGTT in overweight/obese subjects, without differences in insulin response (Study 1).

Although our previous results support the hypoglycemic effect of an amino acid supplement in rats and human, the effects of this supplement on blood glucose response, muscle glycogen resynthesis, muscle damage, and subsequent anaerobic performance post strenuous exercise have not been evaluated in athletes. Therefore, the objective of the present investigation was to study the effects of an amino acid mixture, composed of isoleucine and 4 additional amino acids, on blood glucose homeostasis and recovery in athletes after strenuous exercise. In addition, we investigated whether the amino acid mixture activates cellular signaling proteins that regulate glucose transport and glycogen synthesis. Moreover, we tested the effects of two different doses of the amino acid mixture in combination with carbohydrate supplementation.

EXPERIMENTAL PROCEDURES

Subjects. Ten healthy active adults (7 men, 3 women) volunteered for the study. Subject characteristics are presented in Table 4.1. Subjects were 27.5±2.0 yr of age, 175.2±2.6 cm in height, and weighed 71.6±2.8 kg. All subjects were accustomed to cycling for prolonged periods of 3-5 hours. Before testing, subjects were given a detailed explanation of the procedures to be used and the potential risks of the study. During the initial visit, volunteers completed *the Informed Consent to Participate in Research* and filled out the *Participation Health Research Screening Form*. The experimental protocol was approved by The University of Texas at Austin Institutional Review Board. Maximum oxygen uptake (VO_{2max}) was measured in all subjects on a cycle ergometer by using a TrueOne 2400 metabolic measurement system (ParvoMedics, Sandy, UT) to verify adequate aerobic fitness levels. The average VO_{2max} was 51.9±1.6 $ml \bullet kg^{-1} \bullet min^{-1}$ in men and 46.4±3.0 $ml \bullet kg^{-1} \bullet min^{-1}$ in women.

Experimental protocol. Two to three days after the VO_{2max} test, the subjects reported to the laboratory to perform a practice ride to familiarize them with the

laboratory environment and the experimental protocol. The practice ride was also used to adjust and verify appropriate workloads for the experimental trials. The practice rides simulated the protocol ride but without blood samples being taken. The ride consisted of cycling at 70% VO_{2max} for 2 hr, which was followed by two 1-min sprints at maximal effort at 85% VO_{2max}. The sprints were separated by 1 min cycling at 45% VO_{2max}. During the first 15 min of each hour, oxygen uptake was measured for 5 min by using the same metabolic measurement system as used during the VO_{2max} test, which was used to verify workload. Water (250 ml) was provided every 20 min of exercise. Heart rate (HR) was monitored and ratings of perceived exertion (RPE) were collected every 30 min of exercise. The practice ride and each of the following three experimental trials were separated by a minimum of 7 days. The subjects performed each trial in a room of 19-21 ℃ at the same time of the day over a 3-week period.

The experimental protocol is presented in Fig. 4.1. On the day of each experimental trial, the subjects reported to the lab in the morning after a 12 hr fast during which time they were allowed to consume only water. On reporting to the laboratory, body weight was obtained and a heart rate monitor (Cardiosport, Deer Park, NY) was secured in place around their chest. A Teflon catheter was inserted into a forearm vein, fitted with a three-way stopcock, extended with a catheter-extension, and taped in place. The catheter was kept patent with regular sterile saline washes. After sitting quietly for 2 min, a resting heart rate was recorded and a blood sample was drawn. The subjects then mounted the ergometer and started a glycogen-depleting cycling bout, consisting of cycling at 70% VO_{2max} for 2 hr, followed by five 1-min sprints at maximal effort at 85% VO_{2max}. Each sprint was separated by 1 min of rest during which time the subject cycled at approximately 45% VO_{2max}. During the exercise trial, the laboratory temperature was maintained at 19-21℃ and two floor fans were directed towards the subject to reduce thermal stress. Water (250 ml) was provided every 20 min of exercise. HR and RPE were recorded every 30 min and at the end of the sprint bout. Constant verbal encouragement was given to the subjects during each trial. Blood sampling and a muscle biopsy were performed immediately on cessation of exercise. After the muscle biopsy, subjects were

given the first of two supplement doses. The timing for recovery was started immediately after complete ingestion of the first supplement. At 120 min into the recovery period, subjects were given the second dose of the supplement. A second muscle biopsy was performed after 240 min of recovery to assess muscle glycogen resynthesis. A questionnaire was completed during recovery to rate the effects of the test supplements on stomach feeling and gastrointestinal distress.

Within 30 min of the second muscle biopsy, subjects performed a Wingate Anaerobic Test (WAnT). The subjects mounted the ergometer and cycled at a self-selected leisurely rate. After a 30 second warm up, subjects began pedaling as fast as possible without any resistance. Within 5 seconds, a fixed resistance (0.098 kg/kg body weight for men, 0.095 kg/kg body weight for women) was applied to the flywheel and subjects continued to pedal "all out" for 30 seconds. An electrical counter continuously recorded flywheel revolutions in 5 second intervals. Twenty hours after WAnT, each subject returned to the lab for the last blood draw.

Nutritional supplementation. Subjects received either a 1.2 g CHO/kg body weight (CHO), 1.2 g CHO/kg body weight plus 6.5 g amino acid mixture (CHO/LAA) or 1.2 g CHO/kg body weight plus 13 g amino acid mixture (CHO/HAA) immediately after the first muscle biopsy and at 120 min into the recovery period in a double-blinded, counter-balanced order. Carbohydrate was obtained as 100 g dextrose in a 10 oz orange flavored drink (SUN-DEX, Fisher Healthcare, Houston, TX) and the amount of drink was calculated according to each subject's body weight in order to provide 1.2 g CHO/kg body weight. CHO/LAA consisted of 0.046g cystine 2HCl, 0.023g methionine, 0.045g valine, 6.342g isoleucine and 0.044g leucine per person and was added to the dextrose drink. CHO/HAA was twice CHO/LAA. The test beverages (CHO, CHO/LAA or CHO/HAA) were similar in color, taste, and texture to allow a double-blinded and counter-balanced study design. A laboratory technician who was not involved in the data collection supplied the drinks to each subject.

Dietary/training control. The subjects were instructed to maintain the same dietary intake and activity during the 48 hr period before each trial. They were also asked

to refrain from intense exercise and alcohol for 24 hr before each trial. In addition, the subjects were asked to fast for 12 hr before reporting to the laboratory, during which they could not eat or drink anything except water. They were also instructed to provide a copy of their training and dietary logs for the 2 days before each experimental trial.

Physiological measures. Using Cardiosport heart rate monitor units (Deer Park, NY), HR was recorded at the beginning of exercise, every 30 min of exercise, at the end of the sprints, and at the end of WAnT. Subjective RPE on a Borg-scale (ranging from 6 to 20) was obtained at the same time points as HR obtained during exercise.

Anaerobic performance. WAnT was used to measure anaerobic leg power. Anaerobic power is defined simply as peak power relative to the subject's body mass. Peak power was ideally measured during the first 5 second interval of WAnT, expressed in Watts. The decline in power divided by test duration is defined as the fatigue index. Anaerobic capacity is estimated as mean power during 30 seconds of the test relative to the subject's body mass. Total work is the total amount of work accomplished in 30 seconds of the test, expressed in Joules. The test occurred after 4 hr recovery from the initial fatiguing exercise bout on the same day.

Blood sample collection and analysis. To quantify the effects of the supplements on glucose metabolism and muscle damage, we assessed the levels of blood glucose, insulin, lactate, creatine kinase (CK), and myoglobin. Blood samples (6 ml) were collected from a catheter inserted in a forearm vein, before the onset of the 2 hr cycling bout, on the cessation of sprint bouts, periodically during recovery and at the end of WAnT. During recovery, blood samples were collected at 30, 120, 150, and 240 min of recovery. Twenty hours after WAnT, each subject returned to the lab for the last blood draw. Five milliliters of each blood sample was put into one 5 ml gold top vacutainer tube containing clot activator and gel for serum separation. A 0.5 ml sample was transferred to another 12x75 mm chilled culture tube containing 1 ml of 10% perchloric acid. Vacutainer tubes were centrifuged for 15 min at 1,300 g and culture tubes were centrifuged for 10 min at 3,000 rpm at 4℃ with a HS-4 rotor in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific Inc. Waltham, MA). Serum and perchloric acid extracts were transferred to separate

eppendorf microcentrifuge tubes and stored at -80℃. Each subject's samples were analyzed in duplicate and concurrently after completion of all trials. Serum extracts were used for analysis of glucose, insulin, CK and myoglobin. Perchloric acid extracts were used for enzymatic analysis of blood lactate.

Plasma glucose was determined using a colorimetric method, which employs glucose oxidase (GOD) and a modified Trinder color reaction (Trinder, 1969). The modified Trinder reagent contains the enzyme peroxidase (HPOD), 4-aminoantipyrine (4-AAP) and p-hydroxybenzene sulfonate (p-HBS). Glucose is oxidized to D-gluconate by GOD with production of equal amount of hydrogen peroxide. Catalyzed by HPOD, 4-AAP and p-HBS are oxidized by hydrogen peroxide and form a quinoneimine dye, intensely colored in red. The absorbance of the reaction solution was measured using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at a wavelength of 500 nm. The intensity of the color in the reaction solution is proportional to the concentration of glucose in the blood sample. Duplicate samples were measured and the intra-assay coefficient of variance was 2.2%.

Serum insulin was measured using commercially available ^{125}I radioimmunoassay (RIA) kits (MP biomedicals, LLC. Solon, OH). In a radioimmunoassay, the serum insulin competes with its ^{125}I-labeled counterpart for a limited and constant number of binding sites on the antibody. The amount of antibody-bound ^{125}I-labeled insulin decreases as the concentration of the serum insulin increases. After incubation, antibody-bound insulin was separated from free ^{125}I-labeled insulin by precipitation and centrifugation. The radioactivity of antibody-bound ^{125}I-labeled insulin was counted in a Wallac 1470 Wizard™ automatic gamma counter (Perkin Elmer life sciences, Turku, Finland). The concentrations of serum insulin were calculated based on prepared standards. Duplicate samples were measured and the intra-assay coefficient of variances for insulin assay was 4.5%.

Lactate was determined spectrophotometrically as described by Hohorst (Hohorst, 1965). Coupled with β-NAD and hydrazine, lactate was oxidized by lactate dehydrogenase (LDH) to generate pyruvate hydrazone and β-NADH. The reaction can be monitored by the changes in the fluorescence of NADH, which was measured using a

Beckman DU 640 spectrophotometer at a wavelength of 340 nm. Duplicate samples were measured and the intra-assay coefficient of variance was 1.3%.

Serum CK was measured spectrophotometrically using a Creatine Kinase Reagent Set (Pointe Scientific, Inc. Canton, MI). CK catalyzes the reversible phosphorylation of ADP to form ATP and creatine in the presence of creatine phosphate. Coupled with the formed ATP, glucose is phosphorylated by hexokinase(HK) to generate ADP and glucose-6-phosphate (G-6-P). The G-6-P is then oxidized by G6PDH to form 6-phosphogluconate with the concomitant production of NADH. This reaction can be monitored by the changes in the fluorescence of NADH, which was measured using a Beckman DU 640 spectrophotometer at a wavelength of 340 nm. Duplicate samples were measured and the intra-assay coefficient of variance was 3.6%.

Myoglobin was measured spectrophotometrically using a Myoglobin Enzyme Immunoassay Test Kit (Bio Check, Inc., Foster City, CA). This test is based on the principle of a solid phase enzyme-linked immunosorbent assay (ELISA). The absorbance of the reaction solution was measured using a Bio-Tek ELx800 microplate reader (Bio-Tek Inc., Winooski, VT) at a wavelength of 450 nm. The intensity of the color in the reaction solution is proportional to the concentration of myoglobin in the blood sample. Duplicate samples were measured and the intra-assay coefficient of variance was 8.0%.

Tissue processing. Muscle biopsies (~50 mg wet wt) were taken immediately after exercise and after 4 hr of recovery according to Bergstrom (Bergstrom et al., 1967) from the vastus lateralis, a muscle highly recruited during cycling. The biopsies were frozen in liquid nitrogen and stored at -80℃ for subsequent determination of glycogen and protein. The muscle samples were weighed and homogenized in 9X volume of ice-cold buffer (pH 7.4), containing 20 mM Hepes, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM glycerolphosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM Benzamidine, and 0.5 mM Na vanadate. The muscle homogenate was then centrifuged at 1,000 rpm for 2 min at 4℃ to collect the entire sample. Half of the homogenate was used for muscle glycogen analysis and the other half for western blotting.

Muscle glycogen analysis. The muscle homogenate samples were transferred and added to 1 ml of ice-cold 1N KOH, which were then incubated at 60℃ for 30 min. After

cool down to room temperature, a 100 µl aliquot of the KOH-digested muscle homogenate was added to 250 µl of 0.3 M sodium acetate (pH 4.8) and 10 µl of 50% of glacial acetate acid. The KOH-digested muscle homogenate was then incubated overnight in 250 µl of 0.3 M sodium acetate buffer (pH 4.8) containing 10 mg/ml amyloglucosidase. After overnight incubation, 25 µl of 1 N NaOH was added to terminate the reaction. Liberated glucose was then measured using a spectophotometric Trinder color reaction (Cliniqa Corporation, San Marcos, CA) (Trinder, 1969). Duplicate samples were measured and the intra-assay coefficient of variance was 0.7%.

Western blotting. Proteins investigated included mTOR, Akt/PKB, Akt substrate of 160 kDa (AS160) and glycogen synthase (GS). The muscle homogenate samples were centrifuged at 14,000 g for 10 min at 4℃ and supernatant then aliquoted to several test tubes for western blotting. A modified version of the Lowry assay (Lowry et al., 1951) was used to determine the protein concentration of the homogenate. The phosphorylation of mTOR, Akt/PKB, AS160 and GS were used as an indirect measurement of their activity. The phosphorylation of a structural protein, α-Tubulin, was used as an internal control. Sample protein (100 µg) was combined with an equal amount (1:1) of sample buffer (1.25M tris, 20% glycerol, 20% SDS, 0.25% bromophenol, β-mercaptoethanol, pH 6.8) and boiled at 95℃ for 5 min. Next, sample proteins were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were separated on a 10% polyacrylamide resolving gel for 2 hr. The resolved proteins were then transferred to a nitrocellulose membrane using a wet transfer unit and blocked in 7% nonfat dry milk in Tris-Tween-buffered saline (NFDM/TTBS) for 30 min at room temperature. The membranes were then incubated with either affinity purified anti-phospho-Akt/PKB (Ser-473), anti-phospho-AS160 (Thr-642), anti-phospho-GS (Ser-641), anti-phospho-mTOR (Ser-2448), or anti-α-Tubulin (Cell Signaling Technology, Inc., Danvers, MA) overnight at 4℃. These phosphorylation sites were chosen as an indirect measurement of activity because they represent the primary sites of phosphorylation of the respective protein under insulin-stimulated conditions. The primary antibodies were diluted to either 1:1000 (phospho-AKT/PKB), 1:800 (phospho-

AS160), 1:1000 (phospho-GS), 1:900 (phosphor-mTOR) or 1:1000 (phosphor-α-Tubulin) in TTBS containing 2% NFDM. Following overnight incubation, the membranes were washed for three, 5-min washes in TTBS then incubated for 1 hr at room temperature with species-specific (anti-rabbit) immunoglobulin G (IgG) secondary antibodies (Cell Signaling Technology, Inc., Danvers, MA). The secondary antibodies were diluted to 1:800 in TTBS containing 2% NFDM. The membranes were washed again for three, 5-min washes with TTBS and the anti-body-bound proteins were visualized using Western Lightning Plus-ECL Enhanced Chemiluminescence Reagent (PerkinElmer, Inc., Waltham, MA) according to the manufacturer's protocol. Images were detected and quantified using Quantity One analysis software (Bio-Rad, Hercules, CA). All samples were run with a control from insulin-stimulated rat skeletal muscle and molecular ladder. The amount of phosphorylated target protein on the membrane was normalized to α-tubulin as a control between samples.

Statistical analysis. Statistical analyses were performed by using SPSS 13.0 software (SPSS Inc, Chicago, IL). The data was analyzed using a two-way ANOVA (treatment x time) with repeated measures. Post hoc analysis was performed when significance was found using Fisher's Least Square Difference (LSD). Paired t-tests were performed to determine if significant differences in the area under the curve (AUC) for glucose and insulin occurred between treatments. Statistical significance was set at $p<.05$. All data was displayed as mean ± standard error (SE).

RESULTS

The mean fasting blood glucose values were not significantly different among the three treatments (CHO 4.6±0.1 mmol/L, CHO/LAA 4.8±0.2 mmol/L, CHO/HAA 4.7±0.1 mmol/L). Immediately post 2 hr strenuous exercise, there was no significant difference in the mean blood glucose values between the treatments (CHO 4.4±0.3 mmol/L, CHO/LAA 4.3±0.3 mmol/L, CHO/HAA 4.2±0.3 mmol/L) (Fig. 4.2A). Within 30 min after ingestion of the first supplement, blood glucose increased significantly regardless of the treatments. However, there was a significant treatment effect with the blood glucose response for the CHO/HAA treatment significantly lower than that of the CHO treatment

during the 4 hr recovery. This result was supported by the finding that the glucose AUC for the CHO/HAA treatment was significantly lower than for the CHO treatment (Fig. 4.2B). Statistical analysis also identified a treatment-by-time interaction with blood glucose during the CHO/HAA treatment lower than the CHO treatment at 150 and 240 min during the recovery. No significant treatment effect was detected between the CHO and the CHO/LAA treatments. However, the glucose AUC for the CHO/LAA treatment was significantly lower than for the CHO treatment. Also, a treatment-by-time interaction was identified with blood glucose during the CHO/LAA treatment lower than the CHO treatment at 240 min during the 4 hr recovery. There was no significant difference between the CHO/HAA and CHO/LAA treatments, nor was there a significant difference in the glucose AUC between the two treatments. There was also no significant difference in the blood glucose post WAnT between all three treatments.

The mean fasting plasma insulin values were not significantly different among the three treatments (CHO 108.8±12.0 pmol/L, CHO/LAA 133.4±26.9 pmol/L, CHO/HAA 111.7±10.3 pmol/L). Immediately post 2 hr strenuous cycling exercise, blood insulin decreased dramatically in all three treatments with no significant difference between the treatments (CHO 62.6±5.8 pmol/L, CHO/LAA 65.6±7.3 pmol/L, CHO/HAA 66.4±11.0) (Fig. 4.3A). After ingestion of the first supplement, insulin levels rose significantly at 30 min and then declined until the second supplement was ingested. Insulin levels then significantly increased again during the next 30 min and declined steadily thereafter. There was a significant treatment effect with the blood insulin response for the CHO/HAA treatment significantly higher than that of the CHO and CHO/LAA treatments during the 4 hr recovery. This result was supported by the finding that the insulin AUC for the CHO/HAA treatment was significantly higher than for the CHO and CHO/LAA treatments (Fig. 4.3B). Statistical analysis also identified a treatment-by-time interaction with blood insulin during the CHO/HAA treatment higher than the CHO treatment at 30, 120 and 150 min, and higher than the CHO/LAA treatment at 30 and 150 min during the recovery. There was no significant difference between the CHO and the CHO/LAA treatments, nor was there a significant difference in the insulin AUC between the two treatments (Fig. 4.3B). There was also no significant difference in the blood insulin post WAnT between all three treatments.

Plasma lactate rose significantly after the first 2 hr cycling bout and post WAnT without significant differences between treatments (Fig. 4.4). During recovery, plasma lactate significantly decreased in all three treatments. There were no differences in plasma lactate between the three treatments at any time point during recovery.

There were no significant differences in muscle glycogen concentration among the three treatments immediately after exercise (CHO 26.3±4.6 μmol/g wet muscle, CHO/LAA 33.5±4.8 μmol/g wet muscle, CHO/HAA 28.5±5.9 μmol/g wet muscle) or after 4 hr recovery (CHO 47.8±4.5 μmol/g wet muscle, CHO/LAA 51.7±5.0 μmol/g wet muscle, CHO/HAA 43.9±5.1 μmol/g wet muscle). After ingestion of the beverages immediately post exercise and 2 hrs post exercise, muscle glycogen concentration significantly increased in all three treatments (Fig. 4.5). Muscle glycogen synthesis rate during 4 hr recovery was significantly greater during the CHO treatment compared with the CHO/HAA treatment. There was no difference in glycogen synthesis rate between the CHO and CHO/LAA treatments, and between the CHO/LAA and CHO/HAA treatments.

The phosphorylation status of insulin-signaling proteins for glucose uptake (AS160, Akt/PKB) and glycogen synthesis (GS, mTOR) was assessed as an indirect measurement of activity level. A structural protein, α-tubulin, was used to normalize the amount of target protein on the membrane as a control between samples. There were no significant differences in AS160 phosphorylation at Thr642 (Fig. 4.6) and in GS phosphorylation at Ser641 (Fig. 4.7) in all three treatments immediately post 2 hr strenuous exercise or post 4 hr recovery. Phosphorylation of AS160 at Thr642 was significantly decreased and phosphorylation of GS at Ser641 was significantly increased post 4 hr recovery regardless of the supplement ingested. Although the phosphorylation of mTOR at Ser2448 did not differ between treatments immediately post 2 hr strenuous exercise, it was significantly higher in the CHO/HAA than the CHO treatment at the end of 4 hr recovery (Fig. 4.8). Meanwhile, the phosphorylation of mTOR at Ser2448 significantly increased in the CHO/HAA treatments, but not in the CHO and CHO/LAA treatments. There were no significant differences in Akt/PKB phosphorylation at Ser473 in all three treatments immediately post 2 hr strenuous exercise or post 4 hr recovery (Fig. 4.9). The phosphorylation of Akt/PKB at Ser473 was significantly increased in the

CHO/HAA at the end of 4 hr recovery, but not in the other two treatments, compared with immediately post exercise.

No differences in serum CK occurred between treatments immediately post 2 hr strenuous exercise or after 24 hr recovery (Fig, 4.10A). Serum CK increased significantly 24 hr post exercise in all three treatments. Similar to the serum CK responses, serum myoglobin concentrations did not differ between the treatments at any time point (Fig. 4.10B). Serum myoglobin levels rose immediately post 2 hr strenuous exercise and continued to increase during the first 2 hours in all three treatments. Immediately after WAnT, serum myoglobin declined slightly regardless of the treatment ingested.

WAnT results are presented in Table 4.2. Total work accomplished within 30 sec sprint was presented in Fig. 4.11. There were no significant differences identified in mean power, peak power, anaerobic capacity, anaerobic power, fatigue index or total work among the three treatments.

DISCUSSION

Amino acid mixture reduces blood glucose response to a carbohydrate supplement postexercise

In the present study, we investigated the acute effect of an amino acid mixture supplement after strenuous exercise on glucose tolerance, muscle glycogen resynthesis, muscle damage markers, subsequent anaerobic exercise performance, and activation states of insulin-signaling proteins involved in glucose uptake and glycogen synthesis. The key finding of the present study is that the amino acid mixture, composed of isoleucine, cystine, methionine, valine, and leucine, can lower the glucose response to a carbohydrate supplement given immediately and 2 hr post strenuous exercise in trained athletes. This result was supported by the finding that the glucose AUC for the CHO/HAA and CHO/LAA treatments was significantly lower than that for the CHO treatment. In addition, we found that the blood glucose response was significantly reduced within 30 min of ingesting the second supplement in the CHO/HAA treatment, and also at 120 min in both the CHO/HAA and CHO/LAA treatments compared with the CHO only treatment. These results are consistent with our previous studies, which show that the amino acid mixture can reduce blood glucose responses to an oral glucose

challenge in Sprague-Dawley rats (Bernard et al., 2011) and in healthy overweight/obese adults (Study 1).

Increased insulin response is partly responsible for the hypoglycemic effect of an amino acid mixture

Some studies ascribe the hypoglycemic effects of amino acids to an increased insulin response (Kalogeropoulou et al., 2008). It is known that protein and certain amino acids, such as leucine, isoleucine, arginine, and methionine, are insulin secretagogues (Boden and Tappy, 1990; Bolea et al., 1997; Fajans et al., 1967; Milner, 1970; Sener and Malaisse, 1981; Sener et al., 1981). Milner et al. (Milner, 1970) reported that both leucine and isoleucine stimulated insulin release from rabbit pancreas incubated in a medium containing 1.5 mg glucose/ml. Leucine has been reported to increase the blood insulin response and reduce the blood glucose response in humans when ingested with carbohydrate (Kalogeropoulou et al., 2008; van Loon et al., 2000). Kalogeropoulou et al. (Kalogeropoulou et al., 2008) reported that 1 mmol/kg lean body mass leucine (a mean of 7 g) provided with 25 g glucose attenuated the blood glucose response and strongly stimulated additional insulin secretion in healthy subjects, compared with a 25 g glucose only treatment. In contrast, Nuttall et al. (Nuttall, 2008) reported that 1 mmol/kg lean body mass isoleucine (a mean of 7.4 g) significantly reduced blood glucose in non-diabetic subjects without changes in plasma insulin compared with placebo. When ingested with 25 g glucose, isoleucine reduced the blood glucose response without a significant rise in plasma insulin area response compared with glucose only treatment. However, they found that the mean insulin concentration was greatly increased when isoleucine was ingested with glucose. The mean insulin concentration increased 8-fold when isoleucine was ingested with glucose, but only increased 5-fold when glucose was ingested alone. When compared to that resulting from glucose only ingestion, the mean insulin concentration increased 49% with isoleucine/glucose. The insulin response increased more rapidly and also decreased more rapidly with isoleucine/glucose compared with glucose only treatment, which caused a similar plasma area response between two treatments. Therefore, it is possible that isoleucine per se has no effect on insulin response but it may synergistically stimulate glucose-induced insulin secretion. In

the present study, the CHO/HAA treatment significantly increased blood insulin response compared with the CHO and CHO/LAA treatments. The amino acid mixture in the CHO/HAA supplement is mainly composed of isoleucine (12.684g/2hr), which is about 1.35 mmol/kg body mass (about 1.72 mmol/kg lean body mass). Leucine is only a small portion (0.088g/2hr), which is about 9.36 μmol/kg body mass. Moreover, it was found that the plasma concentration of isoleucine was over threefold that of leucine 1 hr after oral administration of the same amount of individual amino acid in rats (Doi et al., 2003). Therefore, the higher insulin response during the CHO/HAA treatment should be due to the insulin-secretion effect of isoleucine but not leucine. Nevertheless, the mechanisms involved for the insulin-secretion effect of isoleucine remain to be determined.

In Study 1, one dose of a high amino acid mixture also decreased the blood glucose response during an oral glucose tolerance test in overweight/obese adults. However, no additional insulin secretion was identified with the amino acid mixture treatment in Study 1. The conflicting findings in Study 1 and the present study may be due to the experimental design. Study 1 gave the amino acid mixture with 100 g-carbohydrate supplement to sedentary overweight/obese adults. The present study gave the amino acid mixture with 1.2g/kg body weight carbohydrate to healthy active adults immediately and 2 hr post a strenuous exercise. The blood insulin levels post supplements are strikingly different in these two studies. The highest insulin levels reached were 1000-1300 pmol/L at 30 min post supplement in Study 1, but only reached 300-400 pmol/L at 30 min after the first supplement and reached 400-600 pmol/L at 30 min after the second supplement in the present study. The reduced insulin response to a carbohydrate supplement post exercise is consistent with many studies, which found that insulin levels are frequently reduced after exercise (Cinar et al., 2008; Jurimae et al., 1990). The lower insulin response post exercise is thought to be a control mechanism to prevent further decrease in blood glucose. Isoleucine may be only capable of stimulating insulin release when the circulating insulin level is low. This is also supported by the study done by Nuttall et al. (Nuttall, 2008). They found that isoleucine ingested with glucose greatly increased the insulin response. The mean circulating insulin concentration increased from 63 pmol/L to a maximum of 333 pmol/L at 40 min after ingestion of glucose. Addition of isoleucine to glucose increased insulin to a maximum of 486 pmol/L

at 40 min. The much higher insulin levels found in Study 1 may conceal the insulin-secretion effect of isoleucine.

Amino acid mixture reduces blood glucose response independently of insulin

As discussed above, the higher insulin response caused by isoleucine may be partly responsible for the lower blood glucose levels during the CHO/HAA treatment. Amino acid mixture also may decrease blood glucose responses independently of insulin. Several animal studies have suggested the insulin-independent hypoglycemic effect of amino acids *in vivo* (Bernard et al., 2011; Doi et al., 2003; Doi et al., 2005; Doi et al., 2007; Ikehara et al., 2008) and *in vitro* (Doi et al., 2003; Kleinert et al., 2011; Nishitani et al., 2002). Nuttall et al. (Nuttall, 2008) reported that 1 mmol/kg lean body mass isoleucine significantly reduced blood glucose in non-diabetic subjects without changes in plasma insulin, indicating an insulin-independent hypoglycemic effect of isoleucine. Bernard et al. (Bernard et al., 2011) from our laboratory recently found that gavaging Sprague-Dawley rats with a similar amino acid mixture reduced blood glucose response to an oral glucose challenge without additional effect on insulin response. Their results indicate that our amino acid mixture lowers blood glucose independently of insulin. In the present study, our results in the CHO/LAA treatment are consistent with an insulin-independent effect of amino acids on control of blood glucose. The glucose AUC for the CHO/LAA treatment was significantly lower than that for the CHO treatment; however, no significant difference in the plasma insulin response was identified between the CHO/LAA and CHO treatments. Therefore, the improved glucose tolerance by the amino acid mixture in the present study may be partly due to amino acid mixture itself and partly due to an increased plasma insulin response.

Amino acid mixture decreased glucose response by inhibiting hepatic glucose output and increasing muscle glucose uptake

There are several possible explanations for the lower blood glucose response after an amino acid mixture ingested with carbohydrate. One is that the gastric emptying may be slowed down by the amino acid mixture, resulting in a smaller blood glucose response. Dietary protein before or with a meal generally slows gastric emptying (Bowen et al.,

2006) and produces greater satiety (Latner and Schwartz, 1999; Ma et al., 2009; Poppitt et al., 1998; Porrini et al., 1995). However, when the amount of supplement ingested is concerned, it is unlikely the small amount of amino acids provided during the present study (13g/2hr for CHO/HAA or 6.5g/2hr for CHO/LAA) would have the same effect on gastric emptying as dietary protein.

Another possibility is that the amino acid mixture suppressed hepatic glucose output. After prolonged exercise, the hepatic glucose output is elevated to maintain overall glucose homeostasis (Ahlborg et al., 1986; Roy and Parker, 2007). The increased glucose output from liver is mainly due to an increase in hepatic gluconeogenesis rather than glycogenolysis since the liver glycogen stores are depleted substantially during the prolonged exercise. The ability of insulin to suppress hepatic glucose output is well accepted (Ader and Bergman, 1990; De Bodo et al., 1959; Rizza et al., 1981; Steele et al., 1965). Several studies suggested that a higher than 600 pmol/L insulin concentration is required to suppress gluconeogenesis completely in human (Adkins et al., 2003; Chiasson et al., 1980; Gastaldelli et al., 2001). In the present study, the highest insulin level observed after supplements is about 400-600 pmol/L. Therefore, the significantly higher insulin levels found in the CHO/HAA treatment may inhibit the hepatic glucose output to a greater extent than the CHO only treatment, resulting in a smaller blood glucose response. Moreover, amino acid mixture itself also may inhibit hepatic glucose output directly. Doi et al. (Doi et al., 2007) reported that under insulin-free conditions, isoleucine significantly inhibited hepatic gluconeogenesis when alanine was used as a glucogenic substrate in isolated hepatocytes. This was accompanied by a reduction in mRNA levels for phosphoenolpyruvate carboxykinase (PEPCK), a key gluconeogenic enzyme, and a decrease in PEPCK activity. A reduction in mRNA levels for glucose-6-phosphatase (G6Pase) and a decreased activity of G6Pase were also found in both isoleucine-incubated hepatocytes and isoleucine-administered rats. These findings suggest that the hypoglycemic effect of amino acids could be due, in part, to a reduced hepatic glucose output.

A third possibility for the hypoglycemic effect of the amino acid mixture in the present study was an acceleration in peripheral tissue glucose uptake. Insulin-stimulated glucose uptake has been well recognized. The higher insulin response in the CHO/HAA

treatment may cause a greater muscle glucose uptake. Moreover, several *in vivo* and *in vitro* animal studies also reported that amino acids improved glucose tolerance independent of insulin, which was associated with an increase in skeletal muscle glucose uptake (Bernard et al., 2011; Doi et al., 2005; Doi et al., 2007; Kleinert et al., 2011). Doi et al. (Doi et al., 2007) found that oral administration of isoleucine (0.45g/kg body weight) with a bolus intravenous administration of 2-deoxyglucose (2-DG) significantly decreased the plasma glucose level by 20% and enhanced skeletal muscle glucose uptake by 71% in rats without a significant elevation of the plasma insulin level compared with controls. In contrast, no significant changes in glucose uptake were identified in the liver or adipose tissue. Moreover, Bernard et al. (Bernard et al., 2011) from our laboratory gavaged Sprague-Dawley rats with either glucose (CHO) or glucose plus a similar amino acid mixture (CHO-AA) as used in the present study. A bolus containing [^{3}H] 2-DG and [U-^{14}C] mannitol was infused via a tail vein 15 min after gavage. They found that muscle glucose uptake was significantly higher in CHO-AA compared with CHO in both fast-twitch red and white muscle with no difference in insulin between treatments. Another recent study from our laboratory also confirmed that the amino acid mixture increased 2-DG uptake in the isolated rat epitrochlearis muscle in the absence of insulin (Kleinert et al., 2011). Furthermore, when the amino acid mixture was combined with a submaximal or maximal insulin concentration, 2-DG uptake was further increased significantly. These results suggest that an increase in skeletal muscle glucose uptake may be contributing to the hypoglycemic effect of the amino acid mixture independently of insulin and its signaling pathway.

The underlying mechanism for isoleucine-induced glucose uptake in skeletal muscles

The mechanism by which amino acids increase muscle glucose uptake is not immediately clear. The possible underlying signaling pathway for amino acid-stimulated glucose uptake in skeletal muscle was investigated in several animal studies (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007; Kleinert et al., 2011; Morifuji et al., 2009; Nishitani et al., 2002). A recent *in vivo* study from our laboratory found that an amino acid mixture significantly elevated phosphorylation of AS160 without altering insulin secretion and Akt/PKB activation stimulated by carbohydrate in Sprague-Dawley rat

skeletal muscles during an oral glucose challenge (Bernard et al., 2011). AS160 is a potential candidate of downstream targets of Akt/PKB and has been suggested to regulate GLUT-4 translocation in response to either insulin or contractile activity (Bruss et al., 2005; Eguez et al., 2005; Larance et al., 2005; Treebak et al., 2006). Another recent study from our laboratory also found that an amino acid mixture significantly increased 2-DG uptake in isolated rat epitrochlearis muscle in the absence or presence of insulin (Kleinert et al., 2011). The amino acid mixture increased phosphorylation of AS160 under these conditions. Moreover, the phosphorylation of Akt/PKB was not affected by the amino acid mixture in the absence or presence of submaximal concentration of insulin. These data suggest that the amino acid mixture may increase glucose uptake in the absence of insulin via AS160 signaling.

In comparison with CHO treatment, insulin was significantly elevated in the CHO/HAA treatment at 120 and 150 min of recovery. This would suggest a greater phosphorylation of AS160 during the CHO/HAA treatment since AS160 is regulated by insulin. Moreover, we found that Akt/PKB phosphorylation was significantly increased after 4 hr recovery in the CHO/HAA treatment but not in the other two treatments. This change in Akt/PKB phosphorylation may be due to the higher insulin response in the CHO/HAA treatment, since isoleucine was found to have no effect on Akt/PKB (Atherton et al., 2010; Bernard et al., 2011; Kimball et al., 1999; Peyrollier et al., 2000). Akt/PKB is suggested to be the upstream protein for insulin simulated phosphorylation of AS160 (Dreyer et al., 2008); however, we did not see a difference in AS160 phosphorylation between treatments in the present study. The reason for the lack of effects of amino acid mixture and insulin on AS160 phosphorylation is not clear. One might be that AS160 phosphorylation was significantly increased and then reversed in the CHO/HAA treatment before the end of 4 hr recovery. Bernard et al. (Bernard et al., 2011) gavaged Sprague-Dawley rats with either 22.5% glucose or our amino acid mixture in 22.5% glucose and sacrificed them 60 min after gavage. AS160 phosphorylation in skeletal muscle was significantly increased at 60 min after the amino acid mixture treatment; however, how long the higher phosphorylation of AS160 can be sustained is unknown. Unfortunately, we only had biopsies immediately post exercise and at the end

of the 4 hr recovery, which may have missed the optimal time frame to detect the effect of the amino acid mixture on AS160 phosphorylation. Regardless, the significantly lower glucose responses during the CHO/HAA and CHO/LAA treatments compared with the CHO treatment, but similar AS160 phosphorylation, may suggest the hypoglycemic effect of the amino acid mixture was mainly due to inhibition of hepatic glucose output and not muscle glucose clearance.

Amino acid mixture directs increased glucose flux in the muscle to pyruvate oxidation rather than glycogen synthesis

Once glucose enters muscle cells, it is rapidly converted to glucose-6-phosphate (G-6-P) by hexokinase. It may then undergo glycolysis to pyruvate or can enter the glycogen synthetic pathway depending on the prevailing conditions (Mandarino et al., 1993). In the present study, the average muscle glycogen synthesis rate during 4 hr recovery was 5.4 μmol/g wet muscle•h^{-1} in the CHO treatment. This rate is similar to the one reported in a study by Zawadzki et al. (Zawadzki et al., 1992) during which subjects ingested 112 g carbohydrate immediately and 2 hr after strenuous exercise and the average glycogen synthesis rate was 5.5 μmol/g wet muscle•h^{-1}. In the present study, the muscle glycogen synthesis rate during 4-hr recovery was significantly lower in the CHO/HAA treatment compared with the CHO treatment. We then assessed the phosphorylation status of GS, which is the key enzyme regulating glycogen synthesis. GS is normally activated by dephosphorylating at $Ser^{641,645}$ by GSK-3 via the insulin/Akt/PKB signaling pathway (Cohen, 1999; Cross et al., 1995). In the present study, we found no significant differences in GS phosphorylation at Ser^{641} among all three treatments immediately post 2 hr strenuous exercise or post 4 hr recovery, suggesting that amino acid mixture had no effect on GS activation. The blood insulin response was significantly higher in the CHO/HAA treatment. Moreover, the phosphorylation of Akt/PKB at Ser^{473} was significantly increased in the CHO/HAA at the end of 4 hr recovery compared with those immediately post exercise, but this did not occur in the other two treatments. These results indicate that the insulin signaling pathway was better activated in the CHO/HAA treatment compared with the CHO treatment. Furthermore,

increased insulin, as well as leucine, also may stimulate the phosphorylation of mTOR, which leads to the phosphorylation and the subsequent activation of p70S6k (Armstrong et al., 2001; Kimball et al., 2002; Peyrollier et al., 2000). It has been reported that activated p70S6k may induce inactivation of GSK-3, which in turn triggers activation of GS (Armstrong et al., 2001; Sutherland and Cohen, 1994; Sutherland et al., 1993). In the present study, we also found a significantly higher phosphorylation of mTOR at Ser^{2448} in the CHO/HAA treatments after 4 hr recovery, but this did not occur in the CHO and CHO/LAA treatments. In addition, the phosphorylation of mTOR was significantly higher in the CHO/HAA compared with the CHO treatment at the end of 4 hr recovery, although it did not differ between treatments immediately post 2-hr strenuous exercise. As a result, the insulin-activated stimuli on GS, via two distinct signaling pathways, may be stronger in the CHO/HAA treatment than the CHO treatment. However, these stimuli may not be sufficient to activate GS and increase muscle glycogen synthesis in the CHO/HAA treatment. By contrast, Morifuji et al. (Morifuji et al., 2009) found that 1 mM leucine-isoleucine dipeptide from whey protein hydrolysates caused a significant stimulation in glucose uptake rate and glycogen storage in isolated epitrochlearis muscles. Leucine has been identified as a strong activator for mTOR while isoleucine has been shown not to have an effect (Anthony et al., 2000; Kimball and Jefferson, 2006). Activated mTOR/p70S6K signaling pathway causes transient inhibition of GSK-3 and subsequent activation of GS, which in turn stimulates muscle glycogen synthesis (Armstrong et al., 2001; Kimball et al., 2002; Peyrollier et al., 2000; Sutherland and Cohen, 1994; Sutherland et al., 1993). Therefore, the increased glycogen storage in the muscle incubated with leucine-isoleucine dipeptide may be due to the activated mTOR signaling by leucine, not isoleucine. In support of this, Doi et al. (Doi et al., 2003) found that 2 mM leucine caused a significant increase in D--[U-^{14}C]-glucose incorporation into intracellular glycogen in C_2C_{12} myotubes, which did not occur with 2 mM isoleucine. Moreover, an oral administration of 1.35 g/kg isoleucine in food-deprived rats significantly decreased the plasma glucose concentration and increased glucose uptake in

the muscle of rats (Doi et al., 2005); however, isoleucine did not increase D-[U-^{14}C]-glucose incorporation into glycogen in the skeletal muscle of rats compared with controls.

In the present study, the muscle glycogen synthesis rate during 4 hr recovery is significantly lower in the CHO/HAA treatment compared with the CHO treatment. This may suggest that the increased glucose flux of the skeletal muscle in the CHO/HAA treatment is directed to glycolysis rather than converted to muscle glycogen, if in fact the lower blood glucose level during the CHO/HAA treatment was due to an increase in muscle glucose uptake. With glycolysis, G-6-P formed from glucose is converted into pyruvate, which can then either be converted to lactate and released from the cell or enter the tricarboxylic acid (TCA) cycle to be oxidized in mitochondria. In the present study, the plasma lactate concentration increased immediately post 2 hr strenuous exercise and steadily decreased during 4 hr recovery and then increased again post Wingate test in all three supplements. Furthermore, there was no significant difference in blood lactate between the treatments at any time point. This may suggest that an increased glucose flux in glycolysis with the CHO/HAA supplement is directed more to pyruvate oxidation rather than lactate formation. Insulin has been shown to activate pyruvate dehydrogenase complex (PDHC), which plays a central role in pyruvate oxidation (Bogardus et al., 1984; Mandarino et al., 1987; Patel and Roche, 1990; Wieland, 1983). One may assume that the significantly higher insulin response in the CHO/HAA treatment may activate PDHC to a greater extent compared with the CHO treatment, resulting in a higher glucose flux entering TCA cycle. Bonadonna et al. (Bonadonna et al., 1993) assessed the effects of insulin on the rates of total glycolysis, glucose oxidation, glycogen synthesis, and glucose recycling in healthy postabsorptive human subjects. They found that physiological hyperinsulinemia (~70 μIU/ml) stimulated glycogen synthesis above baseline more than glycolysis. Considering the higher insulin response in the CHO/HAA treatment without a corresponding higher muscle glycogen synthesis, the effect of insulin on glucose oxidation might have been minimal.

Furthermore, Doi et al. (Doi et al., 2007) reported that isoleucine administration stimulates glucose uptake in the muscle, which was mainly oxidized immediately after exercise. In their study, oral administration of isoleucine (0.45g/kg body weight) with a

bolus intravenous administration of 2-DG significantly decreased the plasma glucose level by 20% and enhanced skeletal muscle glucose uptake by 71% in rats without a significant elevation of the plasma insulin level compared with controls. They then oral gavaged rats with the same amount of isoleucine or saline again and injected a bolus of 30 μCi/kg body wt of [U-^{14}C]glucose. The rats were placed in acryl metabolic cages and normal air was drawn through the cages at 8 l/min. The expired CO_2 over a 30-min period was collected periodically after glucose infusion. They found that expiratory excretion of $^{14}CO_2$ from [U-^{14}C]glucose was increased by 19% in isoleucine-administered rats compared with controls. Haesler et al. and Tappy et al. (Haesler et al., 1994; Tappy et al., 1992) also reported that amino acid infusion increases glucose oxidation in healthy lean humans during exogenous infusion of glucose. In support of the stimulatory effect of isoleucine on glucose oxidation, Doi et al. (Doi et al., 2005) reported that oral administration of isoleucine caused a significant decrease in AMP content and AMP:ATP ratio in the skeletal muscle of rats compared with control. Correspondingly, they also found that isoleucine significantly decreased AMP-activated protein kinase (AMPK) isoform α2 activity, which is dependent on the cellular AMP content (Stapleton et al., 1996; Stein et al., 2000). AMPKα2 is expressed predominantly in the liver and skeletal muscle. The activity of another isoform of AMPK, α1, which is widely expressed, was unchanged in rats administered isoleucine compared with the controls. Doi's work indicates that isoleucine may increase the availability of ATP independent of activation of AMPK in skeletal muscle of rats (Doi et al., 2005). Therefore, isoleucine may increase glucose uptake in the skeletal muscle with the glucose mainly oxidized to improve the cellular energy state in the muscle. The signaling pathway underlying the stimulatory effect of isoleucine on glucose oxidation is still unknown. Further investigations are required to elucidate the mechanism behind this effect. Based on the results from Doi et al. (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007), we speculated that isoleucine stimulates glucose oxidation in the skeletal muscle via an insulin- and AMPK-independent signaling pathway.

Time-dependent exercise-induced activation of proteins controlling glucose transport and glycogen synthesis

It is worth mentioning that AS160 phosphorylation was significantly decreased and GS phosphorylation was significantly increased in all three treatments after 4 hr recovery compared with immediately post exercise values. To our knowledge, this is the first study to demonstrate that exercise-stimulated AS160 phosphorylation decreased at the end of 4 hr recovery with carbohydrate supplementation. The underlying mechanism of decreased AS160 phosphorylation in the present study is not clear. AS160 serves as a converging point for both insulin- and muscle contraction-dependent signaling pathways on glucose uptake in skeletal muscle (Dreyer et al., 2008). AS160 phosphorylation allows the conversion of an inactive GDP-bound Rab protein to its activated GTP-bound form, enabling GLUT4 translocation to cell membrane (Watson and Pessin, 2006). In the present study, the blood glucose concentration returned back to fasting level in all three treatments at the end of 4 hr recovery, suggesting that postprandial blood glucose was completely disposed in the body. However, the blood insulin concentration was still significantly higher at the end of 4 hr recovery compared with the preexercise level in both CHO and CHO/HAA treatments, indicating that the decreased AS160 phosphorylation was not due to a reduction in insulin. A prior bout of moderate-intensity exercise has been shown to immediately phosphorylate AS160 in lean subjects, and this effect was sustained for up to 3 hr in the postexercise period (Howlett et al., 2008; Sriwijitkamol et al., 2007). However, a decrease in AS160 phosphorylation was observed after 4 hr recovery in the present study, suggesting a decline in glucose transport previously stimulated by muscle contraction. This may imply the glycogen status in the skeletal muscle plays a role in regulating glucose uptake process. Exercise-depleted muscle glycogen is replenished over time, resulting in the stimulus for glucose uptake to wear off. To support this hypothesis, an inverse relationship between muscle glycogen concentration and both insulin- (Cartee and Holloszy, 1990; Jensen et al., 1997) and contraction-stimulated (Hespel and Richter, 1990) glucose transport has been reported. In addition, GS phosphorylation was significantly increased after 4 hr recovery regardless of the supplement ingested in the present study, which means inactivation of GS. GS is a rate-determining enzyme for glycogen synthesis with a hierarchal multisite

phosphorylation mechanism, with dephosphorylation increasing GS activity. Dephosphorylation of GS is stimulated by both insulin and muscle contraction (Aschenbach et al., 2001). However, the increase in GS phosphorylation at the end of 4 hr recovery was most likely not a result of a fall in blood insulin as insulin levels still remained significantly elevated above basal. Similar to AS160, insulin- and contraction-stimulated GS activation is strongly influenced inversely by glycogen content (Jensen et al., 2006; Lai et al., 2007). These results suggest that the muscle glycogen content may inversely regulate both glucose uptake and glycogen synthesis, which helps to prevent either insufficient glycogen storage or glycogen overload in the exercised muscle.

Amino acid mixture has no effects on muscle damage markers and subsequent anaerobic exercise performance

In the present study, we measured serum myoglobin during the 4 hr recovery and serum CK at 24 hrs post exercise to assess muscle damage. The increases in serum CK and myoglobin levels indicate structural damage to the muscle cell resulting in the leakage of proteins out of the cell and into the circulation (Peake et al., 2005). In our study, serum CK increased significantly 24 hr post exercise in all three treatments. Serum myoglobin levels were elevated immediately post 2 hr strenuous exercise and continued to increase during the first 2 hours in all three treatments. Immediately after WAnT, serum myoglobin declined slightly regardless of the treatment ingested. These results indicate that prolonged intense exercise causes muscle damage. Moreover, no differences in serum myoglobin and CK occurred among treatments at any time point tested. This may indicate that the amino acid mixture post exercise has no benefit with regards to exercise-induced muscle damage. Previous studies found that daily supplementation or acute ingestion of BCAA may prevent muscle damage that occurs as a result of a prolonged, intense endurance exercise or strength training (Blomstrand and Newsholme, 1992; Coombes and McNaughton, 2000; Greer et al., 2007; Jackman et al., 2010; Negro et al., 2008; Shimomura et al., 2010; Skillen et al., 2008). Discrepancy between our results and previous findings might exist due to administration of the amino acid mixture. First, previous studies examined the effect of BCAA supplementation on muscle damage, which were mainly composed of leucine (Shimomura et al., 2010). Secondly, previous

studies evaluated the chronic effect of daily BCAA supplementation with supplementation lasting 14 days (Coombes and McNaughton, 2000; Skillen et al., 2008). Thirdly, supplementation was provided before and during exercise and not just post exercise (Greer et al., 2007). In the present study, we examined the acute effect of an amino acid mixture, mainly composed of isoleucine, provided with carbohydrate and given immediately and 2 hr post exercise. The different experimental designs may explain the different findings in the present study compared with previous studies.

After 4 hr recovery, subjects underwent an WAnt test. There were no differences identified in anaerobic power, anaerobic capability, fatigue index, and total work accomplished between the three treatments. This is consistent with our muscle damage results. Exercise-induced muscle damage (EIMD) could cause an immediate and prolonged reduction in muscle function, including reductions in muscle force production and power generation. WAnT has been shown to be a highly reliable and applicable test for predicting performance in both individual and team sporting events (Nottle and Nosaka, 2007; Sinnett et al., 2001). Because the external load during WAnT remains constant, a reduction in peak power is the direct result of an inability to achieve a high pedal frequency against a constant resistance. No differences in muscle damage markers and WAnT measures were found between treatments in the present study. This suggests that the amino acid mixture had no significant effect on muscle damage repair or subsequent anaerobic performance. WAnT is composed of a 30 sec all-out cycling sprint and evaluates anaerobic performance. Anaerobic power is the peak power reached relative to body mass and is the output of the ATP-PC energy system, which is ideally measured during the first 5 second interval of WAnT. Anaerobic capacity is the average power output during the entire test relative to body mass and is the combined ability of ATP-CP and glycolytic systems. Previous studies showed that WAnt metabolism is highly anaerobic, with 80% of the energy turnover derived from glycolysis (Beneke et al., 2002). In the present study, no significant difference was found in blood lactate at the end of WAnt between all three treatments, indicating that the amino acid mixture had no significant effect on the anaerobic glycolytic system.

High dosage of amino acid mixture is more effective on blood glucose regulation and activation of insulin-signaling proteins

In the present study, we tested the effects of two different doses of amino acid mixture given immediately and 2 hr post exercise. The CHO/LAA consisted of 0.046 g cystine 2HCl, 0.023 g methionine, 0.045 g valine, 6.342 g isoleucine and 0.044 g leucine per person and CHO/HAA was twice CHO/LAA. There was no gastrointestinal distress reported for any treatments although amino acids have been reported to cause problems in some individuals. Both CHO/HAA and CHO/LAA decreased glucose response compared with CHO treatment. The hypoglycemic effect was greater in CHO/HAA than CHO/LAA treatment [CHO/HAA vs. CHO (p = .016), CHO/LAA vs. CHO (p = .047)]. The CHO/HAA treatment significantly increased the insulin response and decreased muscle glycogen storage rate compared with the CHO treatment while no significant differences were identified between CHO/LAA and CHO treatments. Moreover, the CHO/HAA treatment had a significant effect on mTOR and Akt/PKB phosphorylation compared with CHO treatment. However, there was no difference in protein phosphorylation between CHO/LAA and CHO treatments. As a result, the CHO/LAA treatment was efficient in regulating glucose homeostasis. A higher dosage of amino acid mixture had greater impact in lowering the glucose response to a carbohydrate bolus, but this may have been due to a combined effect of increased insulin response and amino acid mixture itself.

In summary, an amino acid mixture, composed of isoleucine and 4 additional amino acids, lowered the glucose response to carbohydrate supplement given immediately and 2 hr post strenuous exercise. A higher insulin response was observed with the CHO/HAA treatment. Insulin and isoleucine may be responsible for the smaller increase in blood glucose concentration during the CHO/HAA and CHO/LAA treatments by inhibiting hepatic glucose uptake and increasing muscle glucose uptake. The lower muscle glycogen storage rate in the CHO/HAA treatment suggests that increased glucose flux in skeletal muscle were directed to oxidation rather than glycogen synthesis. There were no significant differences in AS160 and GS phosphorylation between treatments immediately post 2 hr strenuous exercise or post 4 hr recovery. Phosphorylation of

mTOR was significantly higher in the CHO/HAA than the CHO treatment after 4 hr recovery. There were no differences in blood lactate, CK, or myoglobin between the three treatments at any time point tested. Moreover, there were no differences in WAnT measures between treatments. This study suggests that a small dosage of amino acid mixture may be effective in controlling blood glucose. A higher dosage of amino acid mixture also may stimulate insulin secretion and activate insulin-induced signaling.

	Age (yr)	Mass (kg)	Height (cm)	VO_2max ($L \cdot kg^{-1} \cdot min^{-1}$)
Mean, n=10	27.5 $\pm$ 2.03	71.63 $\pm$ 2.80	175.2 $\pm$ 2.59	50.23 $\pm$ 1.60
Males, n=7	26.43 $\pm$ 2.15	74.36 $\pm$ 3.38	177.29 $\pm$ 3.13	51.88 $\pm$ 1.63
Females, n=3	30 $\pm$ 5.03	65.27 $\pm$ 2.90	170.33 $\pm$ 3.93	46.38 $\pm$ 3.03

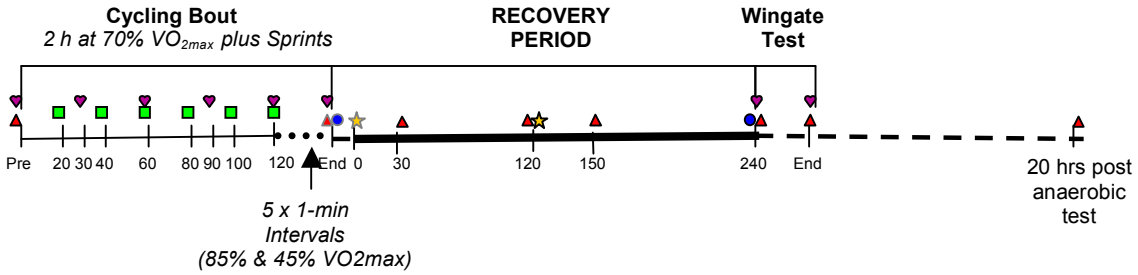
Values are expressed as means + SE.

Table 4.1 Characteristics of subjects

	Mean Power (watts)	Peak Power (watts)	Anaerobic Capacity (w/kg)	Anaerobic Power (w/kg)	Fatigue Index (w/s)	Total Work (joules)
CHO	553.7±23.0	846.4+43.0	7.8+0.2	11.9+0.4	15.2+1.5	16611.8+689.5
CHO/LAA	550.2+20.6	828.2+44.9	7.8+0.2	11.7+0.4	14.8+1.4	16508.5+619.0
CHO/HAA	556.2+23.7	861+47.5	7.8+0.2	12.1+0.4	15.1+1.4	16684.6+710.4

Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. N=10. Values are expressed as means ± SE.

Table 4.2 The Wingate Anaerobic Test (WAnt) results.



Biopsy

Blood collection

Supplement

250ml water

RPE & HR

FIGURE 4.1. EXPERIMENTAL PROTOCOL

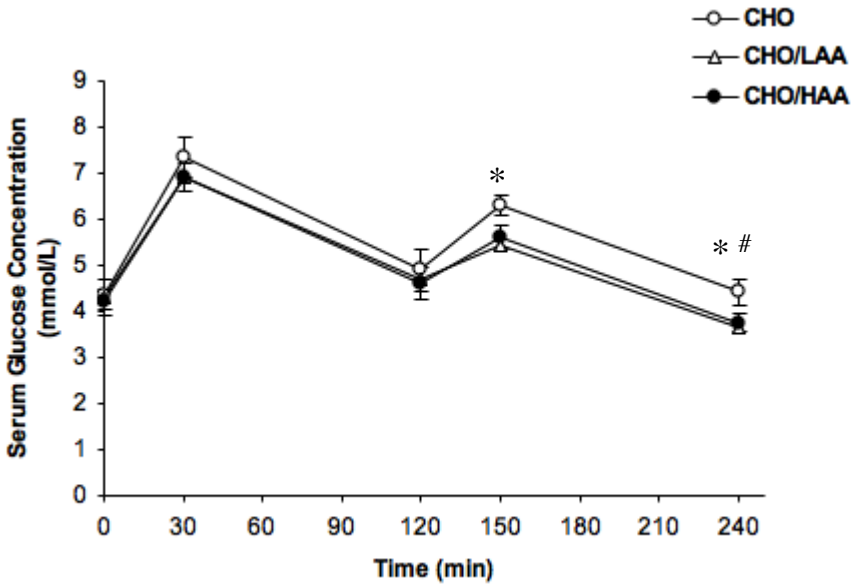


FIGURE 4.2A. BLOOD GLUCOSE POST EXERCISE AND DURING 4 HR RECOVERY. Treatments were with CHO (○), CHO/LAA (△), and CHO/HAA (●) supplements provided immediately after and 2 hr after exercise. Values are means ± SE. *, CHO/HAA vs. CHO ($p < .05$). #, CHO/LAA vs. CHO ($p<.05$).

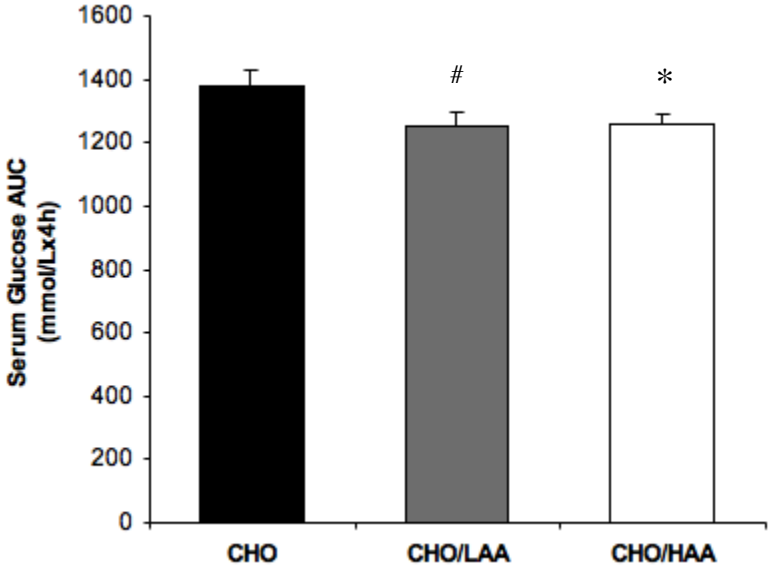


FIGURE 4.2B. BLOOD GLUCOSE AREA UNDER THE CURVE (AUC) DURING 4 HR RECOVERY. Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. AUC was calculated with baseline (pre). Values are means ± SE. *, CHO/HAA vs. CHO ($p < .05$). #, CHO/LAA vs. CHO ($p < .05$).

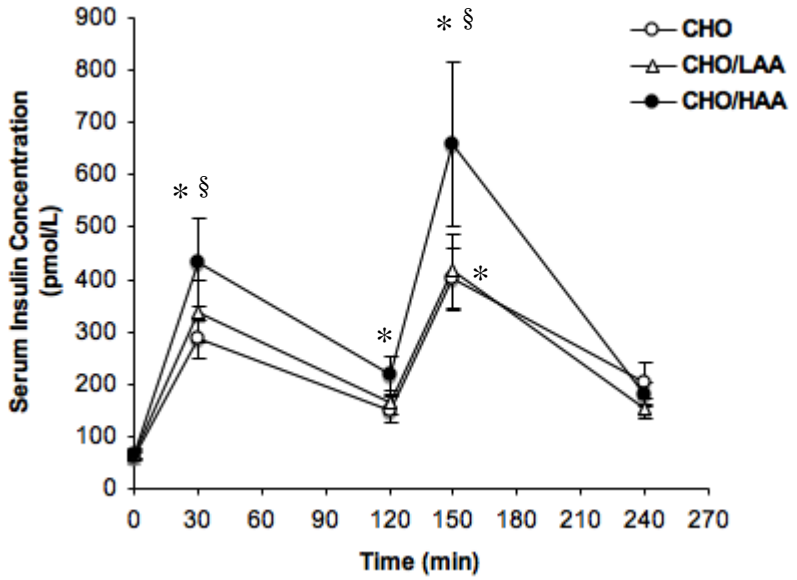


FIGURE 4.3A. BLOOD INSULIN POST EXERCISE AND DURING 4 HR RECOVERY. Treatments were CHO (○), CHO/LAA (△), and CHO/HAA (●) supplements provided immediately after and 2 hr after exercise. Values are means ± SE. *, CHO/HAA vs. CHO ($p < .05$). §, CHO/HAA vs. CHO/LAA ($p < .05$).

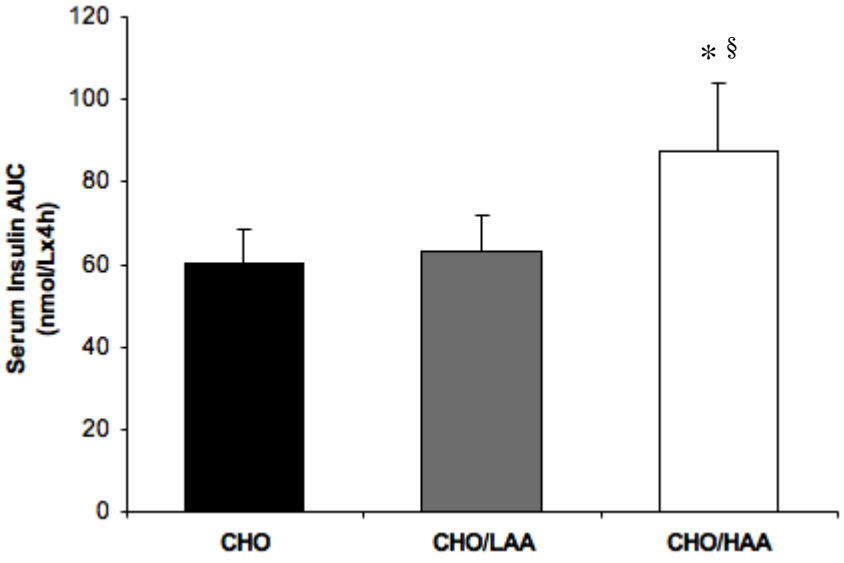


FIGURE 4.3B. BLOOD INSULIN AREA UNDER THE CURVE (AUC) DURING 4 HR RECOVERY. Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. AUC was calculated with baseline (pre). Values are means ± SE. *, CHO/HAA vs. CHO ($p < .05$). §, CHO/HAA vs. CHO/LAA ($p < .05$).

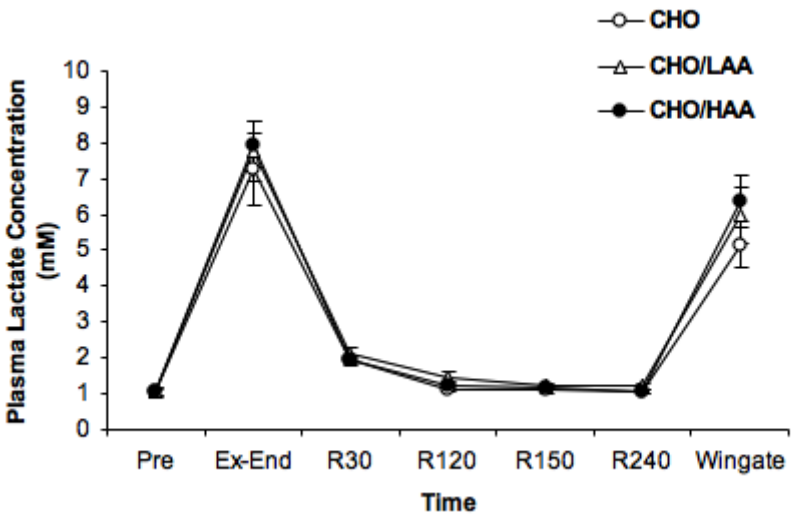


FIGURE 4.4. BLOOD LACTATE DURING EXERCISE AND 4 HR RECOVERY. Treatments were CHO (○), CHO/LAA (△), and CHO/HAA (●) supplements provided immediately after and 2 hr after exercise. Values are means ± SE.

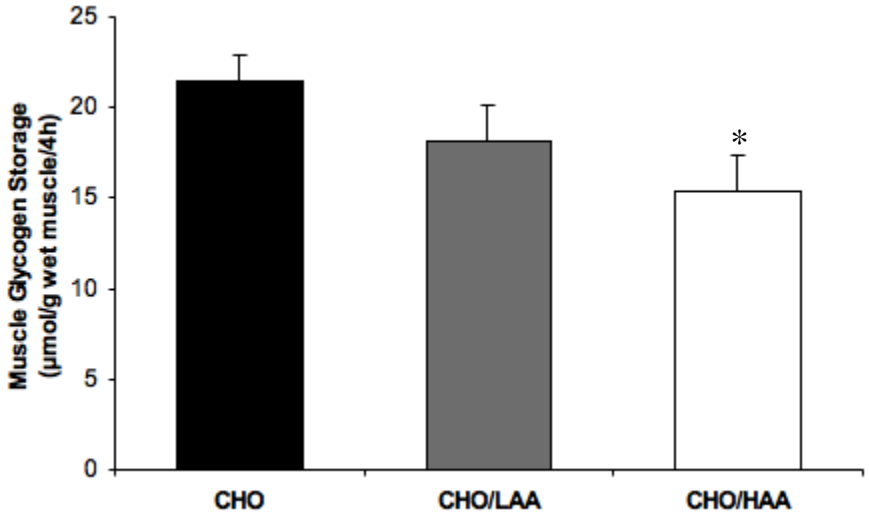


FIGURE 4.5. TOTAL MUSCLE GLYCOGEN STORAGE IN THE VASTUS LATERALIS DURING 4 HR RECOVERY FROM INTENSE CYCLING. Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. Values are means ± SE. *, CHO/HAA vs. CHO ($p < .05$).

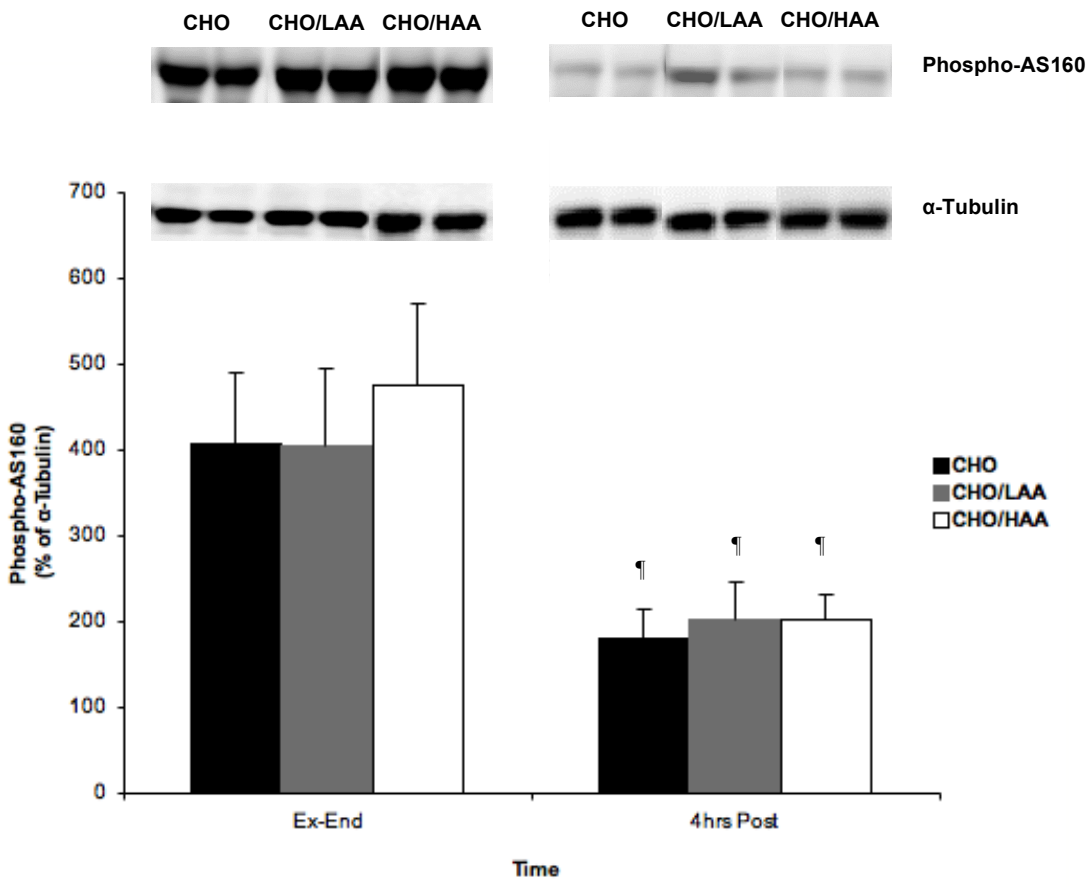


FIGURE 4.6. AS160 THR[642] PHOSPHORYLATION immediately post 2 hr intense cycling (Ex-End) and post 4 hr recovery (4hrs Post). Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. The phosphorylation status of AS160 was assessed as an indirect measurement of its activity level. The amount of phosphorylated AS160 on the membrane was normalized to α-tubulin as a control between samples. Values are means ± SE. ¶, 4hrs Post vs. Ex-End ($p < .05$).

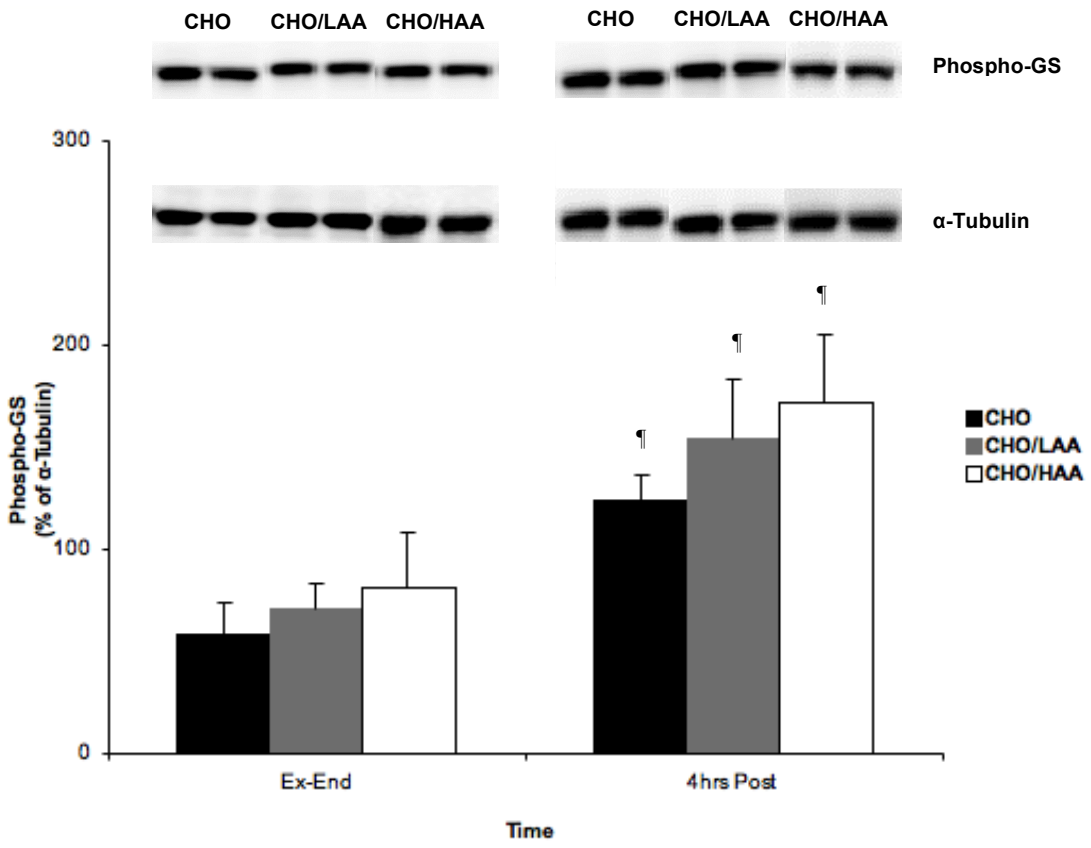


FIGURE 4.7. GLYCOGEN SYNTHASE (GS) SER[641] PHOSPHORYLATION immediately post 2 hr intense cycling (Ex-End) and post 4 hr recovery (4hrs Post). Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. The phosphorylation status of GS was assessed as an indirect measurement of its activity level. The amount of phosphorylated GS on the membrane was normalized to α-tubulin as a control between samples. Values are means $\pm$ SE. ¶, 4hrs Post vs. Ex-End ($p < .05$).

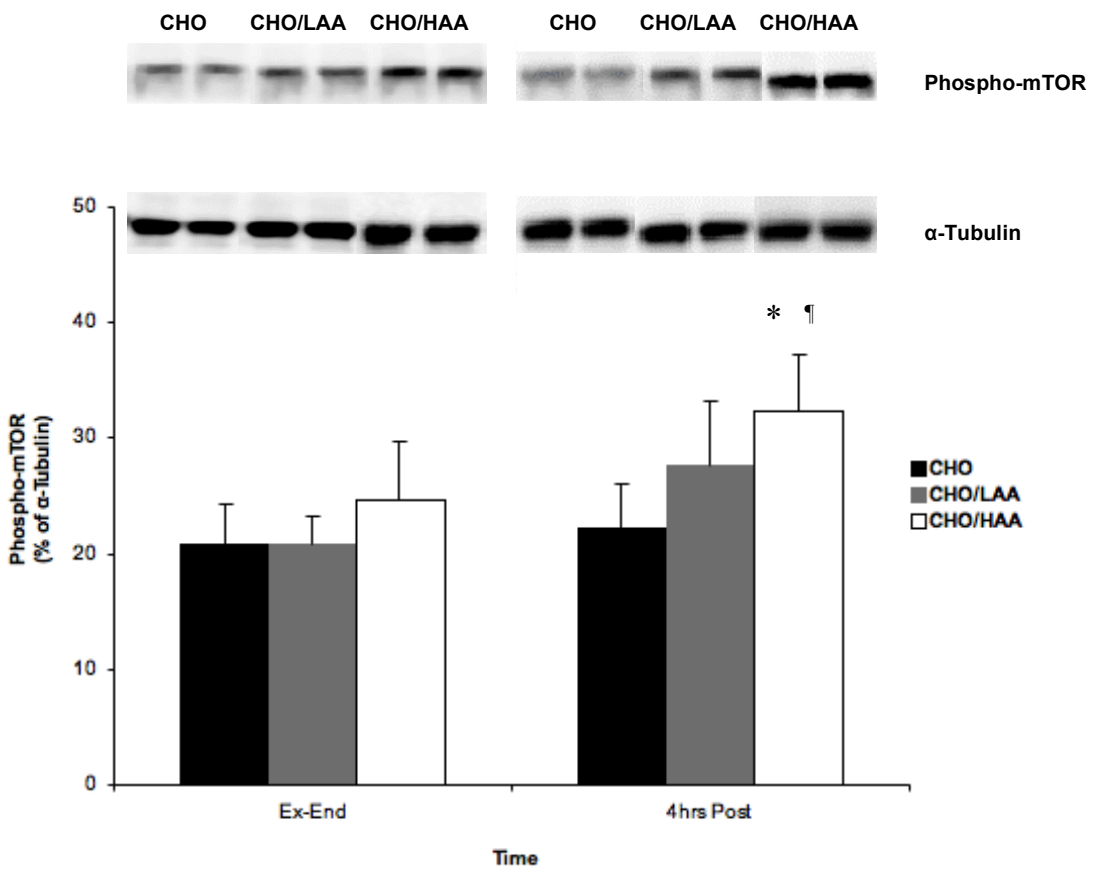


FIGURE 4.8. MTOR SER^{2448} PHOSPHORYLATION immediately post 2 hr intense cycling (Ex-End) and post 4 hr recovery (4hrs Post). Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. The phosphorylation status of mTOR was assessed as an indirect measurement of its activity level. The amount of phosphorylated mTOR on the membrane was normalized to α-tubulin as a control between samples. Values are means $\pm$ SE. *, CHO/HAA vs. CHO ($p < .05$). ¶, 4hrs Post vs. Ex-End ($p < .05$).

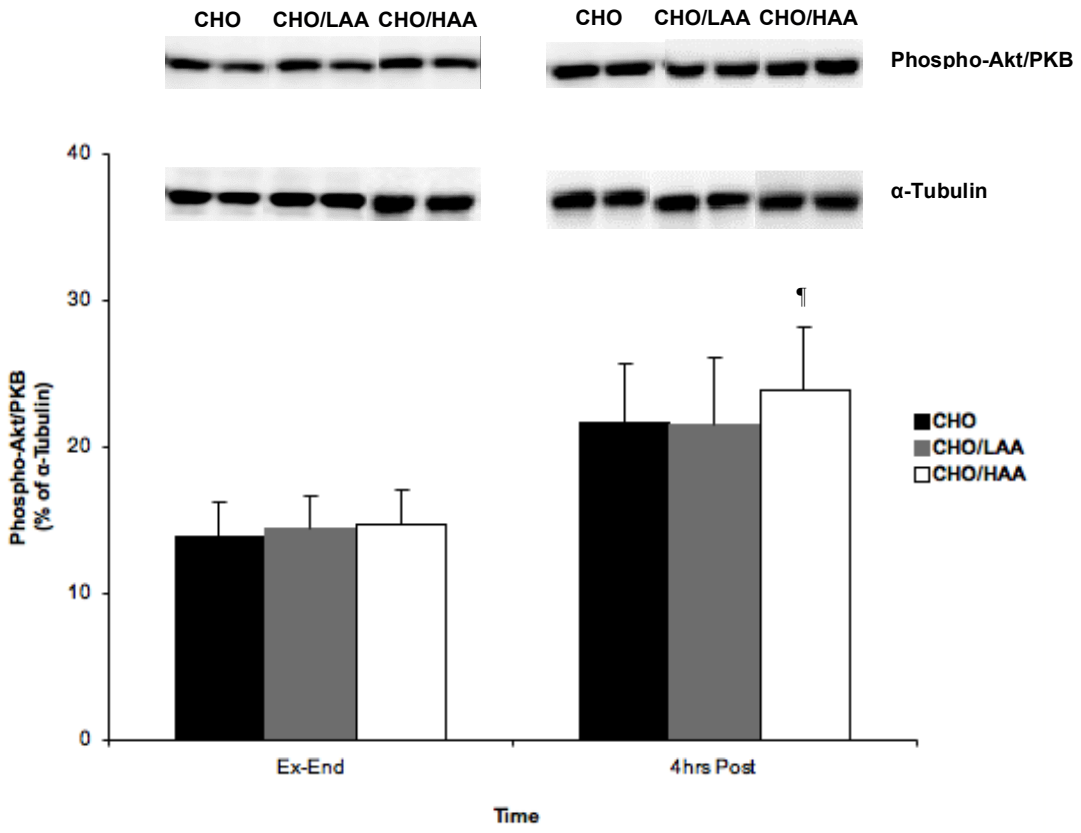


FIGURE 4.9. AKT/PKB SER[473] PHOSPHORYLATION immediately post 2 hr intense cycling (Ex-End) and post 4 hr recovery (4hrs Post). Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. The phosphorylation status of Akt/PKB was assessed as an indirect measurement of its activity level. The amount of phosphorylated Akt/PKB on the membrane was normalized to α-tubulin as a control between samples. Values are means $\pm$ SE. ¶, 4hrs Post vs. Ex-End ($p < .05$).

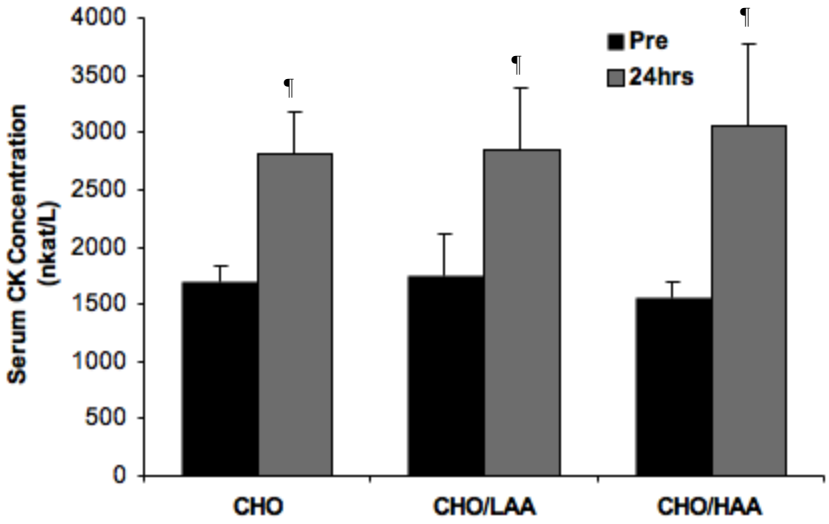


FIGURE 4.10A. SERUM CREATINE KINASE CONCENTRATION BEFORE INTENSIVE CYCLING AND POST 24 HR RECOVERY. Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. Values are means ± SE. ¶, 4hrs Post vs. Ex-End ($p < .05$).

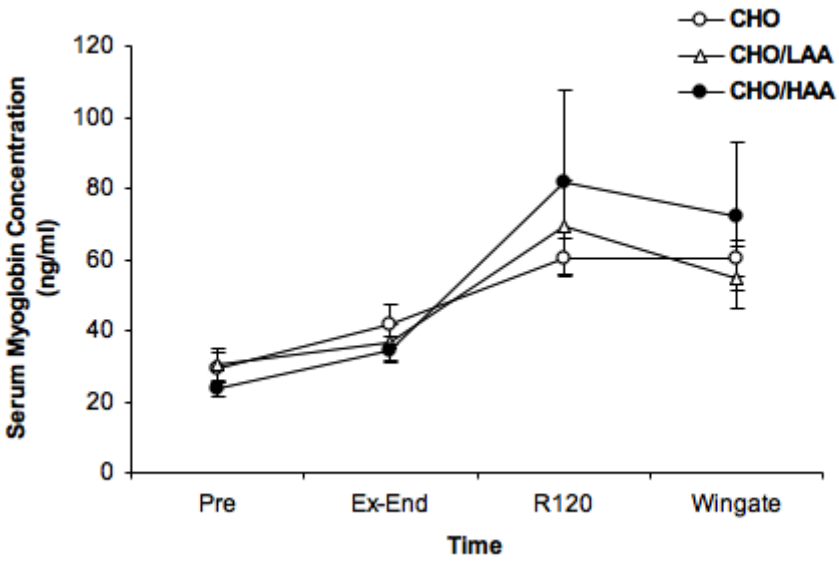


FIGURE 4.10B. SERUM MYOGLOBIN CONCENTRATION DURING EXERCISE AND 4 HR RECOVERY. Treatments were CHO (○), CHO/LAA (△), and CHO/HAA (●) supplements provided immediately after and 2 hr after exercise. Values are means ± SE.

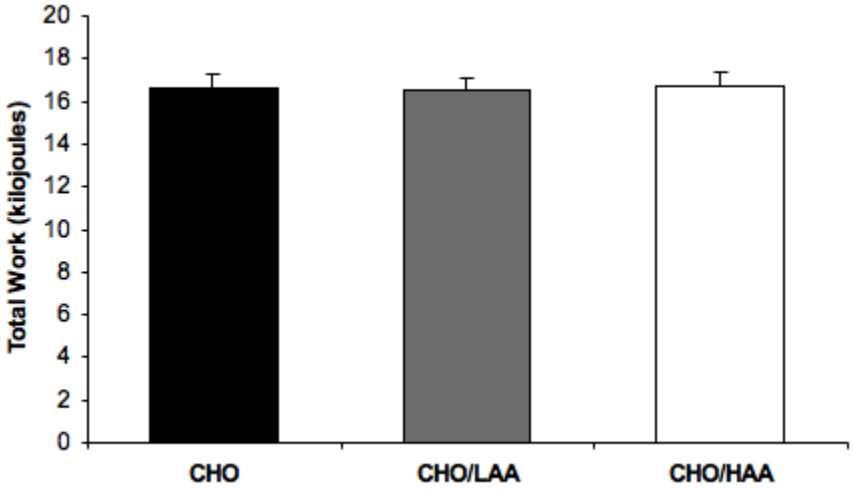


FIGURE 4.11. TOTAL WORK DURING THE WINGATE ANAEROBIC TEST. Treatments were CHO, CHO/LAA, and CHO/HAA supplements provided immediately after and 2 hr after exercise. Values are means $\pm$ SE.

Chapter V: General Discussion

Recent research suggests that amino acids, such as leucine and isoleucine, may lower the blood glucose level by accelerating glucose uptake in peripheral tissues (Bernard et al., 2011; Doi et al., 2003; Doi et al., 2005; Doi et al., 2007; Kalogeropoulou et al., 2008; Kleinert et al., 2011; Morifuji et al., 2009; Nishitani et al., 2002).

Nishitani et al. (Nishitani et al., 2002) reported that 2 mM leucine promoted glucose uptake in soleus muscles isolated from normal rats under insulin-free conditions. Doi et al. (Doi et al., 2003) evaluated the effects of leucine and isoleucine (0.3 g/kg body weight) in normal rats during the oral glucose tolerance test. Isoleucine significantly reduced plasma glucose response to the glucose bolus, whereas leucine did not cause a significant decrease. The measurement of plasma concentration of individual amino acid showed that plasma isoleucine concentration was elevated to 0.89 mM, over threefold that of leucine, which was only 0.27 mM. This may explain the lack of effect of leucine on glucose response in this study. These results indicate that isoleucine is more potent than leucine in lowering blood glucose, which is also supported by the findings that a significant increase in 2-DG uptake occurred when C_2C_{12} myotubes was incubated with isoleucine ranging between 1-10 mM but only happened when incubated with leucine greater than 5 mM. Some investigators have explored the possible underlying pathway for the hypoglycemic effect of amino acids in animal (Doi et al., 2003; Nishitani et al., 2002). It was found that stimulation of glucose uptake by leucine/isoleucine *in vitro* was mediated by PI3K and PKC, but independent of mTOR. The significant hypoglycemic effect of leucine/isoleucine was identified in C_2C_{12} myotubes (Doi et al., 2003), L6 myotubes, and isolated epitrochlearis muscle (Morifuji et al., 2009; Nishitani et al., 2002) in the absence of insulin, and was also shown in normal rats without apparent increase in plasma insulin (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007). The ability of amino acids to attenuate the blood glucose response to a glucose challenge independently of insulin holds great clinical significance for individuals with insulin resistance.

A series of studies have been carried on in our laboratory to explore the effect of an amino acid mixture, composed of isoleucine, leucine, cystine 2HCl, methionine, and

valine, on glucose metabolism and underlying signaling mechanism. A previous study showed that the amino acid mixture is more potent than isoleucine or leucine alone in regulating blood glucose (unpublished). Bernard et al. (Bernard et al., 2011) gavaged Sprague-Dawley rats with either glucose (CHO) or glucose plus the amino acid mixture (CHO-AA). Glucose uptake was significantly higher in CHO-AA compared with CHO in both fast-twitch red and white muscles with no difference in insulin between treatments. A follow-up *in vitro* study confirmed that the amino acid mixture increased 2-DG uptake in the isolated rat epitrochlearis muscle in the absence of insulin (Kleinert et al., 2011). Furthermore, when the amino acid mixture was combined with a maximal insulin concentration, 2-DG uptake was further increased significantly. These results suggest that the amino acid mixture lowers blood glucose by increasing skeletal muscle glucose uptake independently of insulin. When the signaling proteins were examined, we found that amino acid mixture significantly elevated phosphorylation of AS160 in Sprague-Dawley rat skeletal muscles during an oral glucose challenge (Bernard et al., 2011). Concurrently, amino acid mixture increased phosphorylation of AS160 in isolated rat epitrochlearis muscle in the absence or presence of insulin (Kleinert et al., 2011). Moreover, the phosphorylation of Akt/PKB was not affected by the amino acid mixture itself or when the mixture was added to submaximal concentration of insulin. AS160 is one potential candidate of downstream targets of Akt/PKB and has been suggested to regulate GLUT-4 translocation in response to either insulin or contractile activity (Bruss et al., 2005; Eguez et al., 2005; Larance et al., 2005; Treebak et al., 2006). These data suggest that the amino acid mixture may increase glucose uptake in the absence of insulin via AS160 signaling.

The animal studies support the hypoglycemic effect of an amino acid mixture independent of insulin. However, there has not been a comprehensive investigation of the impact of amino acid ingestion on glucose homeostasis in humans. Therefore, one primary objective of this dissertation was to investigate the effects of an amino acid mixture on blood glucose regulation in different populations, including overweight/obese adults and trained athletes.

Once glucose enters muscle cells, it is rapidly phosphorylated and enters glycolysis or glycogen synthetic pathways depending on the prevailing conditions

(Mandarino et al., 1993). The storage form of glucose in the muscles, glycogen, is an important fuel source for moderate to high intensity exercise. It has been clearly demonstrated that aerobic endurance is directly related to the initial muscle glycogen stores (Bergstrom et al., 1967; Bergstrom and Hultman, 1966) and that strenuous exercise cannot be maintained once these stores are depleted (Bergstrom et al., 1967; Bergstrom and Hultman, 1966; Hermansen et al., 1967). Therefore, amino acid supplementation during the recovery period may be beneficial for rapid and effective replenishment of muscle glycogen and subsequent exercise performance, taking account of the hypoglycemic effects of amino acids.

Another important aspect of endurance exercise recovery is muscle damage repair. This damage does not just occur during exercise, but can continue after exercise for many hours. Muscle damage occurs when there is a negative net protein balance, which normally happens immediately after prolonged exercise if no supplement is provided. The ingestion of carbohydrate and protein or amino acids following exercise has been reported to promote protein synthesis and hasten recovery post-exercise, taking advantage of an enhanced insulin response, energy availability, and substrates for protein synthesis (Biolo et al., 1997; Koopman et al., 2005; Levenhagen et al., 2001; Tipton et al., 1999). Based on these studies in which an anabolic response was promoted by carbohydrate-protein/amino acids supplementation, it could be predicted that such supplementation would also reduce exercise-induced muscle damage. Muscle damage could affect force production and result in a reduction in the subsequent anaerobic exercise performance. Therefore, anaerobic performance during a subsequent exercise bout maybe affected by carbohydrate-protein/amino acids supplementation.

Therefore, the secondary objective of this dissertation was to investigate the effects of an amino acid mixture on muscle glycogen synthesis, markers of muscle damage, and subsequent anaerobic performance in athletes. In addition, we examined whether this amino acid mixture can better activate cellular signaling proteins that regulate glucose transport and glycogen synthesis.

Because it has been reported that amino acids cause gastrointestinal distress in some individuals, the third objective of these dissertation studies was to test two different dosages of amino acid mixture and evaluates whether the lower dosage would have a

similar effect on glucose homeostasis as the higher dosage but without gastrointestinal distress.

STUDY 1

In Study 1, the primary aim was to determine if an amino acid mixture lowers the blood glucose response to an oral glucose challenge in healthy overweigh adults. The overweight/obese population is chosen because that weight is considered the single strongest predictor of type 2 diabetes. A summary of results for study 1 is listed in Table 5.1.

Briefly, in Study 1, twenty-two healthy overweight/obese men and women between 20 and 45 years old completed two oral glucose tolerance tests (OGTT) after consuming two different beverages using a double-blinded, random ordered experimental design. The CHO/AA beverage consisted of an amino acid mixture in a 355 ml (12 oz) orange-flavored solution. The amino acid mixture consisted of 0.088 g cystine 2HCl, 0.043 g methionine, 0.086 g valine, 12.094 g isoleucine and 0.084 g leucine per 355 ml solution. The CHO beverage consisted of a 355 ml orange-flavored placebo drink. Before each trial, both beverages were mixed with a 296 ml (10 oz) orange-flavored drink containing 100 g dextrose to make a drink totaling 651 ml. All subjects were instructed to finish the drinks within a total of 15 min. Subjects rested quietly in a room with dimmed lighting during each trial. Venous blood samples (7 ml) were drawn 10 min before the start of ingesting the drinks. Once the drinks were ingested, subsequent blood samples (7 ml) were taken at 15, 30, 60, 120 and 180min.

There were no significant differences in fasting blood glucose levels among the treatment groups. After the completion of experimental drinks, the blood glucose response peaked at 30 min for both treatments, and returned to the fasting level after 120 min during the CHO/AA treatment, but during the CHO treatment this required 180 min. The blood glucose response and glucose AUC for the CHO/AA treatment were significantly lower than that of the CHO treatment. Moreover, the blood glucose during the CHO/AA treatment was lower than the CHO treatment at 30, 60, 120, and 180 min after supplement ingestion. These data suggests that the amino acid mixture lowers blood glucose response to a glucose bolus in healthy overweight/obese subjects.

Insulin is one of major hormones regulating blood glucose. Therefore, the plasma insulin response during the OGTT was determined to assess if it is contributing to the lower glucose response observed with CHO/AA treatment. Plasma c-peptide response was also determined since it serves as an important linker between two chains of insulin and released as equimoler amount as insulin to the portal circulation. The mean fasting plasma insulin values were similar between the two treatments. After ingestion of the beverage, both blood insulin and c-peptide increased immediately and remained higher than the fasting value for the next 180 minutes regardless of treatment received. Furthermore, there was no significant difference in plasma insulin responses between the CHO and CHO/AA treatments, nor was there a significant difference in the insulin AUC between the two treatments Similar to the plasma insulin response, plasma c-peptide concentrations did not differ between the treatments at any time point. These data suggests that the amino acid mixture lowers blood glucose response to a glucose bolus, which was not likely due to an increased plasma insulin response. Our results are consistent with previous *in vivo* and *in vitro* animal studies.

Glucagon is another important hormone regulating blood glucose. The mean fasting plasma glucagon values were not significantly different between the two treatments. However, the blood glucagon concentration was significantly higher at 60 min post CHO than that post the CHO treatment. There was also a significant difference in the glucagon AUC between the two treatments. The relative higher glucagon response in CHO/AA, as compared to the CHO treatment, may be due to the reduced glucose concentration caused by the amino acid mixture. The significantly higher plasma concentration of glucagon at 60 min post CHO/AA corresponded with significantly lower blood glucose concentration in CHO/AA treatment. Moreover, amino acids have been found to stimulate glucagon secretion, which may be partly responsible for the higher glucagon response with CHO/AA treatment. It is worth mentioning that the blood glucose concentration was significantly lower in CHO/AA compared with the CHO treatment, although the plasma glucagon level was higher in CHO/AA and it raises blood glucose levels. These results, therefore, further support the hypoglycemic effect of the amino acid mixture.

There were no significant differences in plasma FFA, TG, or lactate responses between the two treatments at any time point, suggesting that the amino acid mixture has little effect on plasma FFA or TG metabolism and anaerobic glycolysis.

In conclusion, study 1 demonstrated that an amino acid mixture, composed of isoleucine and 4 additional amino acids, lowered the glucose response to an OGTT in healthy overweight/obese subjects. Insulin was not likely responsible for the hypoglycemic effect of amino acid mixture. This study suggests that a mixture of amino acids may be effective in improving the control of blood glucose in individuals at risk of developing type 2 diabetes.

STUDY 2

The primary aim of Study 2 was to study the effects of two different doses of amino acid mixture on blood glucose homeostasis and recovery in athletes after strenuous exercise. In addition, we investigated whether the amino acid mixture activates cellular signaling proteins that regulate glucose transport and glycogen synthesis.

Ten healthy active adults between 18 and 35 years old volunteered for the study. Maximum oxygen uptake (VO_{2max}) was measured in all subjects and the average VO_{2max} was 51.9±1.6 $ml \bullet kg^{-1} \bullet min^{-1}$ in men and 46.4±3.0 $ml \bullet kg^{-1} \bullet min^{-1}$ in women. Each subject completed three experimental trials, during which they had a glycogen-depleting cycling bout, consisting of cycling at 70% VO_{2max} for 2 hr, followed by five 1-min sprints at maximal effort at 85% VO_{2max}. Blood sampling and a muscle biopsy were performed immediately on cessation of exercise. After the muscle biopsy, subjects were given the first of two supplement doses. Subjects received either a 1.2 g CHO/kg body weight (CHO), 1.2 g CHO/kg body weight/6.5 g amino acid mixture (CHO/LAA) or 1.2 g CHO/kg body weight/13 g amino acid mixture (CHO/HAA). Carbohydrate was obtained as 100 g dextrose in a 10 oz orange flavored drink and provided 1.2 g CHO/kg body weight. CHO/LAA consisted of 0.046 g cystine 2HCl, 0.023 g methionine, 0.045 g valine, 6.342 g isoleucine and 0.044 g leucine per dose and was added to the dextrose drink. CHO/HAA was twice CHO/LAA. The timing for recovery was started immediately after complete ingestion of the first supplement. At 120 min into the recovery period, subjects

were given the second dose of the supplement. A second muscle biopsy was performed after 240 min of recovery to assess muscle glycogen resynthesis. Within 30 min of the second muscle biopsy, subjects performed a Wingate Anaerobic Test (WAnT) to assess anaerobic exercise performance. Twenty hours after WAnT, each subject returned to the lab for the last blood draw. A summary of results for study 2 is listed in Table 5.2.

Blood glucose significantly increased within 30 min after ingestion of the first dosage regardless of the treatments. The blood glucose response and glucose AUC for the CHO/HAA treatment were significantly lower than that of the CHO treatments during the 4hr recovery. No significant treatment effect was detected between the CHO and the CHO/LAA treatments. However, the glucose AUC for the CHO/LAA treatment was significantly lower than for the CHO treatment. Moreover, the blood glucose during the CHO/HAA treatment was significantly lower than the CHO treatment at 150 and 240 min during the recovery. Also, the blood glucose during the CHO/LAA treatment was significantly lower than the CHO treatment at 240 min during the 4 hr recovery. These data suggest that amino acid mixture lowers blood glucose response to a glucose bolus post exercise in athletes, with higher dosage more potent than lower dosage.

There were no significant differences in the mean fasting blood insulin or the blood insulin immediately post 2 hr exercise between the three treatments. There was no significant treatment effect between the CHO and the CHO/LAA treatments, nor was there a significant difference in the insulin AUC. This result suggests that CHO/LAA lowers blood glucose response independent of insulin, which is consistent with Study 1.

However, the CHO/HAA treatment caused a significantly higher blood insulin response than that of the CHO and CHO/LAA treatments during the 4 hr recovery. The higher insulin response in the CHO/HAA may be partially responsible for the lower glucose response observed. Both leucine and isoleucine are insulin-secretagogues. However, the amino acid mixture in the CHO/HAA supplement is mainly composed of isoleucine and leucine is only a small portion. Therefore, the higher insulin response during the CHO/HAA treatment should be due to the insulin-secretion effect of isoleucine but not leucine.

In Study 2, significantly higher insulin concentrations were identified at 30 min after first dose and second dose in the CHO/HAA compared with CHO treatment. These results are different with Study 1, which gave subjects the same amount of amino acid mixture (one dose) and did not produce different insulin response between treatments. The different insulin response to amino acid mixture between Study 1 and the current study may be due to the experimental design. In Study 1, the peak insulin reached 1000-1300 pmol/L at 30 min, but in Study 2, it only reached 300-400 pmol/L at 30 min after the first supplement and reached 400-600 pmol/L at 30 min after the second supplement of CHO/HAA. The insulin-secretagogue effect of isoleucine may be limited when the circulating insulin level is getting physiologically high.

The increased insulin response observed in CHO/HAA treatment may increase muscle glucose uptake and suppress hepatic glucose output, which in turn contributes to the lower blood glucose. Moreover, isoleucine has also been found to inhibit hepatic gluconeogenesis in isolated hepatocytes under insulin-free conditions (Doi et al., 2007). Whether isoleucine has a similar effect in humans needs further investigation. Furthermore, previous studies found that isoleucine stimulates glucose uptake in skeletal muscles and the phosphorylation of AS160. However, in Study 2, we did not find any significant differences in AS160 phosphorylation between treatments immediately post 2 hr strenuous exercise or post 4 hr recovery. The reason for the lack of effect of amino acid mixture and insulin on AS160 phosphorylation is not clear. One might be that AS160 phosphorylation was significantly increased and then reversed in the CHO/HAA treatment before the end of 4 hr recovery. In the present study, the insulin level was significantly higher in the CHO/HAA at 120 and 150 min of recovery, indicating that a greater phosphorylation of AS160 may happen in the same time points since AS160 is regulated by insulin. As a result, it is possible that isoleucine lowers blood glucose during a glucose challenge via both insulin-dependent and -independent pathways, which may operate synergistically in stimulating muscle glucose uptake and suppressing hepatic glucose output.

In Study 2, the muscle glycogen synthesis rate during 4 hr recovery was significantly lower in the CHO/HAA treatment compared with the CHO treatment.

Moreover, no significant difference in GS phosphorylation at Ser^{641} was found between all three treatments, suggesting amino acid mixture has no effect on GS activation. In contrast, both Akt/PKB and mTOR were better activated in CHO/HAA compared with CHO treatment. Isoleucine has been shown to have no effect on mTOR. However, the significantly higher insulin response and leucine in the CHO/HAA treatment could theoretically stimulate mTOR phosphorylation, which in turn could activate p70S6k and subsequently inactivate GSK-3, resulting in activation of GS. However, these stimuli from insulin and leucine were apparently insufficient to activate GS during the CHO/HAA treatment.

The lower blood glucose levels and muscle glycogen storage during 4 hr recovery in the CHO/HAA treatment may indicate a higher glycolysis in the muscle. In Study 2, there was no significant difference in blood lactate between the treatments at any time point. This may suggest that an increased glucose flux in glycolysis with the CHO/HAA supplement is directed more to pyruvate oxidation rather than lactate formation. Insulin has been shown to activate pyruvate dehydrogenase complex (PDHC), which plays a central role in pyruvate oxidation (Bogardus et al., 1984; Mandarino et al., 1987; Patel and Roche, 1990; Wieland, 1983). However, insulin was reported to affect glycogen synthesis more than glycolysis. Considering the higher insulin response in the CHO/HAA treatment without corresponding higher muscle glycogen synthesis, the contribution of insulin to possibly increased glucose oxidation in Study 2 may be minimal. Some studies suggested that isoleucine administration stimulates glucose oxidation in rats and healthy lean humans. Moreover, isoleucine has been found to decrease AMP:ATP ratio and increase availability of ATP in rats muscle, which is independent of AMPK activation. Therefore, it is possible that isoleucine may increase glucose uptake in the skeletal muscle with the incorporated glucose mainly oxidized to improve the cellular energy state in the muscle. The signaling pathway underlying the stimulatory effect of isoleucine on glucose oxidation is still unknown, further investigations are required to elucidate the mechanism behind this effect.

In Study 2, we measured serum myoglobin during the 4 hr recovery and serum CK at 24 hrs post the 2 hr strenuous exercise to assess muscle damage. No differences in

serum myoglobin and CK occurred at any time point tested among treatments. After 4 hr recovery, WAnT was performed in all subjects to assess anaerobic exercise performance. Similar to muscle damage markers, there were no differences identified in anaerobic power, anaerobic capability, fatigue index, and total work accomplished within 30 sec sprint between all three treatments. This may indicate that the amino acid mixture post exercise has no additional benefit on exercise-induced muscle damage recovery and the subsequent anaerobic exercise performance. WAnT is composed of a 30 sec all-out cycling sprint and is highly anaerobic, with 80% of the energy turnover is derived from glycolysis. In the present study, no significant difference was found in blood lactate at the end of WAnt between all three treatments. This may indicate that the amino acid mixture has no significant effect on anaerobic metabolism.

In Study 2, we tested the effects of two different doses of amino acid mixture given immediately and 2 hr post exercise. The amount of amino acid mixture in CHO/HAA was twice CHO/LAA. There was no any gastrointestinal distress reported in subjects after all treatments. Both CHO/HAA and CHO/LAA decreased glucose response compared with CHO treatment, with the hypoglycemic effect greater in CHO/HAA than CHO/LAA treatment. The CHO/HAA treatment significantly increased the insulin response and decreased muscle glycogen storage rate compared with CHO treatment while no significant differences were identified between CHO/LAA and CHO treatments. Moreover, the CHO/HAA had a significant effect on mTOR and Akt/PKB phosphorylation compared with CHO treatment. However, there was no difference in protein phosphorylation between CHO/LAA and CHO treatments. As a result, the CHO/LAA was efficient in regulation of glucose homeostasis. The higher dosage of amino acid mixture had a greater impact in lowering glucose response to a carbohydrate bolus, which may be due to a combined effect of increased insulin response and amino acid mixture itself.

In conclusion, an amino acid mixture, composed of isoleucine and 4 additional amino acids, lowers the glucose response to carbohydrate supplement given immediately and 2 hr post strenuous exercise. A higher insulin response was observed with the CHO/HAA treatment. Insulin and isoleucine may be responsible for the smaller increase

in blood glucose concentration during the CHO/HAA and CHO/LAA treatments by inhibiting hepatic glucose uptake and increasing muscle glucose uptake. The lower muscle glycogen storage rate in the CHO/HAA treatment indicates that increased glucose flux in skeletal muscle may be directed to oxidation rather than glycogen synthesis. There were no significant differences in AS160 and GS phosphorylation between treatments immediately post 2 hr strenuous exercise or post 4 hr recovery. Phosphorylation of mTOR was significantly higher in the CHO/HAA than the CHO treatment after 4 hr recovery. There were no differences in blood lactate, CK, or myoglobin between the three treatments at any time point tested. Moreover, there were no differences in WAnT measures between treatments. This study suggests that a small dosage of amino acid mixture may be effective in controlling blood glucose. A higher dosage of amino acid mixture may have more effect on stimulating insulin secretion and maintaining energy status of the cell.

subjects	Intervention	Time	Blood Glucose Level	Blood Insulin Level	Blood C-Peptide Level	Blood Glucagon Level	Blood Lactate Level	Blood FFA Level	Blood TG Level
Healthy Overweight /Obese Subjects	High AA ingested with 100 g glucose	once	decrease	no difference	no difference	increase	no difference	no difference	no difference

Table 5.1 Summary for Study 1 Results. All of the measures are compared with CHO treatment.

subjects	Intervention	Time	Blood Glucose Level	Blood Insulin Level	Blood Lactate Level	Muscle Glycogen Storage Rate	AS 160 Phospho-rylation	Akt/PKB Phospho-rylation	mTOR Phospho-rylation	GS Phospho-rylation	Blood CK & Myoglobin Level	Anaerobic Exercise Performance
Healthy Active Adults	High AA ingested with 1.2g CHO /kg body wt. post exercise	Twice	decrease	increase	no difference	no difference	no difference	no difference vs. CHO Higher vs. basal	increase	no difference	no difference	no difference
	Low AA ingested with 1.2g CHO /kg body wt. post exercise	Twice	decrease	no difference	no difference	no difference	no difference	no difference	no difference	no difference	no difference	no difference

Table 5.2 Summary for Study 2 Results. All of the measures are compared with CHO treatment if not specially mentioned.

FUTURE DIRCTIONS

This series of studies demonstrated the beneficial effects of an amino acid mixture on glucose tolerance in both healthy overweight/obese adults and trained athletes. These studies have also addressed some possible mechanisms underlying the hypoglycemic effect of amino acid mixture. Moreover, these studies evaluated the acute effect of the amino acid mixture on exercise-induced muscle damage and subsequent anaerobic exercise performance. However, several areas warrant further investigation.

The most elusive aspect of these investigations is to fully understand the exact mechanism(s) through which amino acid mixture lowers blood glucose response. Study 2 suggests the hypoglycemic effects of amino acid mixture may be due to the dual regulation of insulin and isoleucine on hepatic glucose output and/or muscle glucose uptake. The use of stable isotope methodology to track the movement of glucose in the body may clarify the mechanism. Furthermore, it was suggested that increased glucose flux in the muscle cell by isoleucine may be directed more to glycolysis rather than glycogen synthesis. A combination of oxygen consumption measurement and stable isotope methodology may provide evidence to support this assumption. If the amino acid mixture does have effects on hepatic glucose output and glucose oxidation, then it would be important to investigate the underlying mechanism responsible for them.

AS160 is the key protein regulating GLUT4 translocation in response to insulin and contraction. Several animal studies have found the involvement of AS160 in isoleucine-induced glucose uptake. However, we did not found any difference in AS160 phosphorylation between treatments in Study 2. One possibility may be that we missed the time phosphorylation of AS160 was elevated. We performed muscle biopsies immediately postexercise and 4 hrs after the first dose of supplement. The phosphorylation of AS160 may be increased after amino acid administration, but quickly reversed within 4 hrs. Therefore, a future study could be designed to examine the effects of amino acid mixture on AS160 phosphorylation at more frequent intervals, which could be done in an animal model.

In addition, Study 2 did not find any benefits with the acute amino acid mixture supplement on exercise-induced muscle damage and subsequent anaerobic exercise

performance. However, daily ingestion of amino acids for at least 14 days has been shown to reduce muscle soreness and muscle damage markers. Therefore, chronic administration of the amino acid mixture may show a significant effect. Moreover, it would be interesting to see whether the chronic administration of amino acid mixture has a better control on blood glucose than an acute dosage.

The amino acid mixture is mainly composed of isoleucine, which has been shown to have no effect on Akt/PKB and mTOR. Insulin-stimulated Akt/PKB and mTOR signaling pathways could inhibit GSK-3 and in turn activate GS, causing an increase in muscle glycogen synthesis. Leucine is potent in insulin secretion stimulation and mTOR activation. However, leucine only accounts for a small portion of our amino acid mixture. Therefore, increasing the leucine proportion in the mixture may cause a greater muscle glycogen synthesis, combined with hypoglycemic effect of isoleucine. A future study could be designed to examine the effectiveness of the amino acid mixture with a higher leucine concentration on muscle glycogen replenishment and subsequent endurance performance.

These studies translated findings from animal studies to healthy humans and determined the effectiveness of amino acid mixture on blood glucose control. However, whether this supplement has a similar effect on impaired glucose tolerance and insulin-resistant populations is not currently known. This could be possibly elucidated by applying the amino acid mixture on these populations during an OGTT. If the amino acid mixture did work in those populations, then it would serve as a non-pharmacological approach to improve blood glucose regulation in diabetes.

Appendix A: Blood Collection and Sample Preparation

Study 1 Immediately upon collection, each blood sample was transferred into two 12 x 75 mm polypropylene culture test tubes cooled on ice, containing ethylenediaminetetraacetic acid solution (EDTA, 24 mg/ml, pH 7.4). From all blood samples, 0.3 ml of the anticoagulated blood was transferred to tubes containing 0.6 ml 10% perchloric acid (PCA). All tubes were centrifuged for 10 min at 3,000 rpm at 4ºC with a HS-4 rotor in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific Inc. Waltham, MA). Following centrifugation, the PCA extracts were transferred to 12 x 75 test tubes and stored for later analysis of blood lactate. The plasma was transferred to five new 12 x 75 test tubes. Three tubes received 0.6 ml plasma each and stored for later analysis of insulin, c-peptide, and glucagon. A protease inhibitor, Trasylol (aprotinin), was added to the glucagon test tubes to prevent proteolysis. A fourth test tube received 0.5 ml plasma and was stored for later analysis of glucose, free fatty acids (FFA), and triglycerides. The remaining plasma was placed in a fifth test tube and saved as a backup. All test tubes were immediately stored at -80° C. Each subject's samples were analyzed in duplicate and concurrently after completion of all trials.

Study 2 Immediately upon collection, five milliliters of each blood sample was put into one 5ml gold top vacutainer tube containing clot activator and gel for serum separation. A 0.5 ml sample was transferred to another 12x75 mm chilled culture tube containing 1 ml of 10% PCA. Vacutainer tubes were centrifuged for 15 min at 1,300 g and culture tubes were centrifuged for 10 min at 3,000 rpm at 4ºC with a HS-4 rotor in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific Inc. Waltham, MA). Serum and the PCA extracts were transferred to separate eppendorf microcentrifuge tubes and were stored at -80℃. Each subject's samples were analyzed concurrently after completion of

all trials. Serum extracts were used for analysis of glucose, insulin, CK and myoglobin. The PCA extracts were used for enzymatic analysis of blood lactate.

Appendix B: Substrate Measurement

(Glucose, Lactate, Free Fatty Acids, Triglycerides)

Glucose Assay

Plasma glucose was determined using Glucose Color Reagent Kits (Cliniqa Corporation, San Marcos, CA), which employs glucose oxidase (GOD) and a modified Trinder color reaction (Trinder, 1969). The modified Trinder reagent contains the enzyme peroxidase (HPOD), 4-aminoantipyrine (4-AAP) and p-hydroxybenzene sulfonate (p-HBS). Glucose is oxidized to D-gluconate by GOD with production of equal amount of hydrogen peroxide. Catalyzed by HPOD, 4-AAP and p-HBS are oxidized by hydrogen peroxide and form a quinoneimine dye, intensely colored in red. The absorbance of the reaction solution was measured using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at a wavelength of 500 nm. The intensity of the color in the reaction solution is proportional to the concentration of glucose in the blood sample. Normal resting, fasted blood glucose levels ranged between 70 and 105 mg/dL.

Three different concentrations of glucose were used as standards and controls to monitor the performance of assay procedures, providing a continued screening of the instrument, reagents and technique. Within and between assay variations was performed on three standards containing varying concentrations of glucose. Data (% CV) shown are from two duplicate determinations of each sample in seven separate assays.

Sample No.	Mean mg/dL	Within % CV	Between % CV
1	50	1.9	9.3
2	100	3.7	7.3
3	200	3.8	2.1

Lactate Assay

Blood lactate was determined spectrophotometrically as described by Hohorst (Hohorst, 1965). Coupled with β-NAD and hydrazine, lactate is oxidized by lactate dehydrogenase (LDH) to generate pyruvate hydrazone and β-NADH. The reaction can be monitored by the changes in the fluorescence of NADH, which was measured using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at a

wavelength of 340nm. All samples were measured in duplicate. Normal resting, fasted blood lactate levels ranged between 0.8 and 1.5 mM. The protocol is below:

Chemicals:

NAD	Sigma N-7004
LDH	Sigma L-3916
Hydrazine	Sigma H-9507
Glycine	Fisher G-7126
Perchloric Acid	Fisher A-229

GLycine-hydrazine Buffer (1000ml):

0.33M glycine	25.02g
0.27M hydrazine	23.94ml

Mix and bring up to 1000ml with dH2O, pH to 9.2.

Procedure:

1. Prepare reagent cocktail

For each sample:

1ml glycine-hydrazine buffer

0.83mg NAD

5U LDH (need 5μl if use 1000U/ml stock)

2. Add 1ml reagent cocktail into test tubes.
3. Add 50μl 10% PCA to test tubes for blank
4. Add 50μl two lactate acid standards to standard test tubes
5. Add 50μl blood samples to sample test tubes
6. Vortex and incubate tubes at 37℃ for 45 minutes in shaking water bath (speed 50 RPM).
7. Warm the spectrophotometer for 30 minutes, read the samples at 340nM.
8. Calculations:

Lactate concentration = (Absorbance/6.22) x 1.05/0.05 x 3/1

Absorbance/6.22 = Absorbance/Absorption Coefficient of NADH (ε = 6.22 cm^{-1} $mmol^{-1}$)

1.05/0.05 = cuvette dilution

3/1 = blood dilution (0.5ml blood in 1ml 10% PCA)

FFA Assay

FFA was measured using a modified colorimetric method stemming from those devised by Ayers (Ayers, 1956) and Iwayama (Iwayama, 1959). FFA is extracted from plasma samples with CHM extraction reagent (choloroform, hepatane, and methanol). Copper nitrate and triethanolamine (TEA) are added to the test solution to form copper soaps of FFA. After centrifugation and separation, color reagent, diethyldithiocarbamate solution dissolved in butanol, was added to the chloroform layer. The color developed was measured using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at a wavelength of 436nm. All samples were measured in duplicate. Normal resting, fasted blood FFA levels ranged from 0.3-0.8 µmol/mL.

Four different concentrations of FFA were used as standards and controls to monitor the performance of assay procedures, providing a continued screening of the instrument, reagents and technique. Within and between assay variation was performed on four standards containing varying concentrations of FFA. Data (% CV) shown are from two duplicate determinations of each sample in 4 separate assays.

Sample No.	Mean µmol/mL	Within % CV	Between % CV
1	0.25	5.5	5.7
2	0.5	5.6	5.9
3	1	4.9	2.7
4	2	5.1	2.1

Triglycerides Assay

Triglycerides were measured using triglyceride GPO reagent (Cliniqa corporation, San Marcos, CA). The triglyceride GPO reagent contained adenosine-5'-triphosphate (ATP), 4-Aminoantpyrine (4-AAP), 3, 5-dichloro-2-hydroxybenzene sulfonate (DHBS), lipases, glycerol kinase (GK), glycerol phosphate oxidase (GPO), and peroxidase (HPOD). TG was hydrolyzed by lipase to glycerol and free fatty acids. Glycerol was phosphorylated by ATP to glycerol-1-phosphate (G-1-P), which was then oxidized to dihydroxyacetone phosphate (DAP) by GPO while producing equal amounts of hydrogen peroxide. Coupled by hydrogen peroxide, 4-AAP and DHBS were oxidized by HPOD and formed a quinoneimine dye, intensely colored in red. The absorbance of the reaction solution was measured using a Cary 50 MPR microplate reader (Varian Australia Pty Ltd, Victoria, Australia) at a wavelength of 520nm. The absorbance of quinoneimine dye in

the reaction solution was proportional to the concentration of TG in the blood sample. Duplicate samples were measured and the intra-assay coefficient of variance was 10.07%.

Appendix C: Blood Hormone Measurement

(Insulin, C-peptide, Glucagon)

Plasma insulin, glucagon, and C-peptide were measured using commercially available ^{125}I radioimmunoassay (RIA) kits. Industry standards and controls are included in the kit. All samples were measured in duplicate. In a radioimmunoassay, the plasma hormone competes with its ^{125}I-labeled counterpart for a limited and constant number of binding sites on the antibody. The amount of antibody-bound ^{125}I-labeled hormone decreases as the concentration of the plasma hormone increases. After incubation, antibody-bound was separated from free ^{125}I-labeled hormones by precipitation and centrifugation. The radioactivity of antibody-bound ^{125}I-labeled hormone was counted in a Wallac 1470 Wizard™ automatic gamma counter (Perkin Elmer life sciences, Turku, Finland). . After determining the average counts per sample, the percentage of radioactive insulin bound to the antibody (%B/Bo) was calculated by the following formula:

%B/Bo = (CPM of standard or sample/ CPM of 0 μIU/mL) x 100

The concentrations of plasma hormones were then calculated using the equation of the standard curve.

Insulin Assay

Plasma insulin was measured using ImmuChem™ Coated Tube ^{125}I RIA Kit (MP biomedicals, LLC. Solon, OH). Insulin levels from healthy, fasting individuals obtained utilizing the ImmuChem™ Insulin RIA kit yielded the following range: 4.3-19.9 μIU/mL (mean = 9.6, n = 24)

Within and between assay variation was shown in the table below.

		Intra-Assay Variation(m=1)			Inter-Assay Variation(m=12)		
Control	n	mean	SD	CV%	Mean	SD	CV%
Pool-1	12	18.19	1.50	8.25	19.07	1.68	8.81
Pool-2	12	36.49	1.54	4.22	35.89	3.04	5.66
Pool-3	12	91.42	4.93	5.39	8.81	8.47	6.36

m=number of assay n=number of determinations

Expected Physiological Insulin Ranges (μIU/mL)

Yalow. R.S. and Berson, S.A., J. Clin. Invest., 39:1157, 1960.
Oral administration of Glucose

	Average Fasting	0.5 hr.	1 hr.	2 hr.	Average
Nondiabetic	21	143	139	106	117
Diabetic	27	97	156	243	147

Merimee, T.J. and Pulkkinen, A.J., J. Clin. Endocrinol. Metab., 45/2, 232, 1977
Oral administration of glucose (100g)

Nondiabetic	Average Fasting	0.5 hr.	1 hr.	2 hr.
Women	12±2	71±7	77±13	59±8.0
Men	11±3	68±14	57±7	51±7.9

McReynolds, C.R., et al. Annals of Clin. Lab Sci., 3/6, 454, 1973
Oral administration of glucose (100g)

	Average Fasting	0.5 hr.	1.5 hr.	2 hr.	3 hr.
Nondiabetic	13	54	54	47	28
Adult onset diabetic	12	67	98	101	53
Moderate severe diabetic	24	43	50	47	33

Glucagon Assay

Plasma glucagon was measured using Millipore Glucagon RIA Kit (Millipore, St. Charles, Missouri. Protocol attached). Normal resting, fasted blood glucagon levels ranged between 50 and 150 pg/mL.

Within and between assay variation was shown in the table below.

Sample No.	Mean pg/mL	Within % CV	Between % CV
1	60	6.8	13.5
2	65	4.0	12.7
3	90	4.6	13.4
4	220	4.0	7.3

C-Peptide Assay

Plasma C-peptide was measured using Millipore Human C-Peptide RIA Kit (Millipore, St. Charles, Missouri. Protocol attached). Normal resting, fasted blood C-peptide levels ranged between 0.5 and 1.5 ng/mL.

Within and between assay variation was shown in the table below.

Sample No.	Mean ng/mL	Within % CV	Between % CV
1	0.4	3.4	9.3
2	0.9	4.4	4.4
3	1.8	6.4	4.2
4	2.1	4.2	3.2
5	2.8	4.5	2.4

Appendix D: Blood Muscle Damage Markers Measurement

(Creatine Kinase, Myoglobin)

Creatine Kinase Assay

Serum creatine kinase (CK) was measured spectrophotometrically using Creatine Kinase Reagent Set (Pointe Scientific, Inc. Canton, MI). CK catalyzes the reversible phosphorylation of ADP to form ATP and creatine in the presence of creatine phosphate. Coupled with the formed ATP, glucose is phosphorylated by hexokinase(HK) to generate ADP and glucose-6-phosphate (G-6-P). The G-6-P is then oxidized by G6PDH to form 6-phosphogluconate with the concomitant production of NADH.

$$\text{ADP + Creatine Phosphate} \xrightarrow{\text{CPK}} \text{Creatine + ATP}$$

$$\text{ATP + Glucose} \xrightarrow{\text{HK}} \text{ADP + Glucose-6-Phosphate}$$

$$\text{G-6-P + } NAD^{+} \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate + NADH + } H^{+}$$

This reaction can be monitored by the changes in the fluorescence of NADH, which was measured using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at a wavelength of 340nm. All samples were measured in duplicate. According to the manufacturer's specifications, within % CV is reported to be 0.9 for 152 determinations, and between %CV is 1.0 for 155 assays.

Calculations

One international Unit (U/L) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under defined conditions.

$$U/L = \Delta Abs./min. \times 1.025 \times 1000 / (1 \times 6.22 \times 0.025)$$

$$= \Delta Abs./min. \times 6592$$

Where: $\Delta Abs./min.$ = Average absorbance change per minute

1.025 = Total reaction volume

1000 = Conversion of U/ml to U/L

1 = Light path in cm

6.22 = Millimolar absorptivity of NADH

0.025 = Sample volume in ml

SI Units: To convert to SI Units (nkat/L) multiple U/L by 16.67

Expected Values

Expected values are based on measurement performed at 37℃. The values for males are up to 160 U/L, and for females are up to 130 U/L.

Myoglobin Assay

Myoglobin was measured spectrophotometrically using Myoglobin Enzyme Immunoassay Test Kit (Bio Check, Inc., Foster City, CA). This test is based on the principle of a solid phase enzyme-linked immunosorbent assay (ELISA). ELISA utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the myoglobin molecule. Mouse monoclonal anti-myoglobin antibody is used for solid phase immobilization on the microtiter wells, while a goat anti-myoglobin antibody is used in the antibody-enzyme conjugate solution. The myoglobin molecules are sandwiched between the solid phase and enzyme-linked antibodies when the test samples react simultaneously with the two antibodies. After incubating at room temperature for 45 minutes, the wells are washed with distilled water to remove unbound labeled antibodies. Then, the wells are incubated with a TMB (Tetrabethyl-benzidine) reagent for 20 minutes, resulting in the development of a blue color. A Stop Solution is added to stop the color development, which changes the color to yellow. The absorbance of the reaction solution was measured using a Bio-Tek ELx800 microplate reader (Bio-Tek Inc., Winooski, VT) at a wavelength of 450 nm. The intensity of the color in the reaction solution is proportional to the concentration of myoglobin in the blood sample. Normal serum myoglobin levels ranged between 12 and 100 ng/ml.

All samples were measured in duplicate. According to the manufacturer's specifications, the lowest detectable level of myoglobin by this assay is estimated to be 5 ng/ml. The within-run and between-run precisions were determined by replicate determinations of five different serum samples.

Intra-assay precision

Serum Sample	1	2	3	4	5
# Reps.	20	20	20	20	20
Mean Myo (ng/ml)	55.6	214.3	294.9	505.9	1,437
S.D.	2.2	12.9	16.2	26.3	94.0
C.V. (%)	3.9%	6.0%	5.5%	5.2%	6.6%

Inter-assay precision

Serum Sample	1	2	3	4	5
# Reps.	35	35	35	35	35
Mean Myo (ng/ml)	59.2	244.4	330.5	568.3	1451.7
S.D.	4.6	12.8	38.9	52.7	104.7
C.V. (%)	7.8%	5.2%	11.8%	9.3%	7.2%

Appendix E: Muscle Biopsy Procedure

On the day before each trial, the biopsy needles were placed in self-seal sterilization pouches (McKesson Corporation, Richmond VA) and sterilized by subjecting them to high pressure steam at 120 °C for 30 minutes using an autoclave (Market Forge Industries, INC). Muscle biopsies (~50 mg wet wt) were taken according to Bergstrom (Bergstrom et al., 1967) from the vastus lateralis, which is a muscle highly recruited during cycling. The thigh was cleansed with 10% betadine solution and then 1.8 ml of a local anesthesia (1% Lidocaine Hydrochloride Injection, Elkins-Sinn, Inc., Cherry Hill, NJ) injected to anesthetize the site of the muscle biopsy using a 25-guage hypodermic needle. A 5-8 mm incision was made through the skin and fascia, 2 inches from the midline of the thigh on the lateral side and 4 inches above the patella. Once the bleeding was stopped, the muscle biopsy was taken using a 3.5 to 5 mm sterile biopsy needle with accompanying suction and pressure was reapplied to the incision to stop bleeding. The biopsy samples were trimmed of adipose and connective tissue and frozen in liquid nitrogen at -80° C for subsequent analysis. Once bleeding stops, the incision was closed with a Band-Aid and a pressure pack was affixed firmly over the incision and taped in place with athletic tape and adhesive wrap. The subjects were given verbal and written instructions on how to care for the biopsy site and were required to return for a recheck and redressing of the site 24 hours later.

Appendix F: Homogenization for Glycogen Measurement and Western Blotting

Muscle biopsies (~50 mg wet wt) were taken immediately after exercise and after 4-h of recovery from the vastus lateralis. The biopsy samples were immediately frozen in liquid nitrogen and stored at -80 ℃ for subsequent determination of glycogen and protein according to Ferguson-Stegall (Ferguson-Stegall et al., 2011). On the day of assay, the muscle samples were weighed and homogenized in 9X volume of ice-cold homogenization buffer (pH 7.4), using 3 x 5 sec bursts with a Caframo RZR1 homogenizer at speed 3-4. The homogenization buffer was made as described below:

Homogenization buffer, pH 7.4 (for 100 mL):

Reagent	Product Number	Formula Weigh	Concentration (mM)	Amount (g)
HEPES	H-3375	238.3	20	0.4766
EGTA	E-4378	380.4	2	0.0761
NaF	S-7920	41.9	50	0.209
KCl	P-217	74.6	100	0.746
EDTA	ED-2SS	292.2	0.2	0.0074
β-Glycerophosphate disodium salt hydrate	G-6251	216.0	50	1.08
DTT	D-0632	154.3	1	0.015
PMSF (Phenylmethanesulfonyl fluride)	P-7626	174.2	0.1	0.00174
Benzamidine	B-6506	156.6	1	0.01566
Sodium orthovanadate	S-6508	181.9	0.5	0.0092

The muscle homogenate was then centrifuged at 1,000 rpm for 2 min at 4℃ to collect the entire sample. Half of the homogenate was used for muscle glycogen analysis immediately. For another half of the homogenate, centrifuge at 14,000 G for 10 min and aliquot the supernatant into 4 x 0.5 mL microcentrifuge tubes per sample. Each aliquot contains ~30 μl of sample supernatant. Freeze them in liquid nitrogen immediately and quickly store in -80^{O}C freezer. Freeze pellet separately at -80^{O}. These are saved for protein quantification and western blotting assays later.

Appendix G: Muscle Glycogen Measurement

SOLUTIONS:

1 N KOH (for 500 mL):
Add 28.01 g KOH (Fisher P-250) into 400 ml ddH_2O, stir on a magnetic plate and bring volume to 500ml with ddH_2O.

0.3 M sodium acetate, pH 4.8 (for 500 mL):
Add 12.3 g sodium acetate (Sigma S-8750) into 300 mL ddH_2O, stir on a magnetic plate, pH to 4.8, bring volume to 500ml with ddH_2O.

50% glacial acetic acid (for 200 mL):
Mix 100 mL glacial acetic acid (Sigma A-9967) with 100 ml ddH_2O, stir on a magnetic plate.

10 mg/ml amyloglucosidase in sodium acetate (for 40 mL):
Add 40 mg amyloglucosidase (Sigma 10115) into 4 mL 0.3 M sodium acetate, pH 4.8 (Sigma S-8750)

1 N NaOH (for 200 mL):
Add 8 g NaOH (Fisher S318-3) into 150 ml ddH_2O, stir on a magnetic plate and bring volume to 200ml with ddH_2O.

PROCEDURE:

1. Add 1 ml of ice-cold 1N KOH into each muscle homogenate tube.
2. Incubate the homogenate tube at 60^oC for 30 min (shaking speed 40 RPM), vortexing vigorously using hand at 20 minute.
3. Transfer 100 µl aliquot of the KOH-digested homogenate to 250 µl of 0.3 M sodium acetate, pH 4.8, and vortex.
4. Add 10µl of 50% of glacial acetate acid to each of the tubes.
5. Add 250 µl of 0.3 M sodium acetate, pH 4.8, containing 10 mg/ml amyloglucosidase to each tube and vortex.
6. Seal tubes with parafilm and incubate overnight at room temperature.
7. The following day, add 25 µl of 1 N NaOH to each sample tube and vortex to terminate reaction.
8. Set up 12 x 75 mm test tubes for Trinder assay (Trinder, 1969) for determination of liberated glucose.
9. Prepare Raichem color reagent according to product insert and pipet 1.5 mL of reagent into each tube, including two standards and samples in duplicate and a blank.
10. Pipet 100 µl of muscle samples into test tubes for the Trinder reaction to begin. Use 2 duplicate glucose standards (4.5 mg and 9.0 mg glucose/dL dH_2O) to run in parallel with the muscle samples.

11. Incubate at 37 ^{o}C for 15 min in a shaking bath set to speed of 50.

CALCULATIONS:

1. (Ab sample / Ab standard) × (standard conc. = 4.5 mg/dl) = mg/dl
2. Dilution factors
   DF1: (1000 µl + half volume in µl of muscle homogenate)/half volume = %
   DF2: [(0.25 ml + 0.01 ml + 0.25 ml + 0.025 ml) + 0.1 ml]/0.1 ml = 6.35
   DF3: volume in cuvette, same in standard = 1
3. Final Calculation:
   [(mg/dl) × half volume in dl of muscle homogenate × DF1 × DF2 × DF3] / half muscle weight (g) = mg glycogen/g wet muscle
   {[(mg glycogen/g muscle)/1000]/180.2}*10^6 = µmol glycogen/g wet muscle

Appendix H: Determination of Muscle Protein Concentration

(Modified Lowry Assay)
(Lowry et al., 1951)

SOLUTIONS:

0.1 N NaOH (1L)
Add 4 g NaOH (Fisher S-318-3) into 900 ml ddH_2O, stir on a magnetic plate and bring volume to 1000ml with ddH_2O.

2% Sodium carbonate (1L)
Add 20 g Sodium carbonate into 900 ml 0.1 N NaOH, stir on a magnetic plate and bring volume to 1000ml with 0.1 N NaOH.

2% Sodium potassium tartrate (200ml)
Add 4 g sodium potassium tartrate (Fisher S-387) into 150 ml ddH_2O, stir on a magnetic plate and bring volume to 200ml with ddH_2O.

1% Cupric sulfate (200ml)
Add 2 g cupric sulfate (Fisher C-489) into 150 ml ddH_2O, stir on a magnetic plate and bring volume to 200ml with ddH_2O.

PROCEDURES:

1. Thaw 1 aliquot of 5 mg/mL BSA (bovine serum albumin) stock and samples on ice.
2. Dilute samples 1:30 by adding 10 µl of supernatant to labeled microcentrifuge tube containing 290 µl ddH_2O and vortex. Keep on ice.
3. Perform serial dilution:

	ddH_2O (ml)	Std protein (ml)	[protein] (mg/ml)
Blank	1.0	0	0
A	1.8	0.2 of BSA Stock	.5
B	0.2	0.8 of A	.4
C	0.5	0.5 of B	.2
D	0.5	0.5 of C	.1
E	0.5	0.5 of D	.05
F	0.5	0.5 of E	.025

4. Make Solution A (1 mL per tube needed) in a 125 mL Erlenmeyer flask:

48 mL	2% Sodium carbonate	Stir with stir bar
1 mL	2% Sodium potassium tartrate	Gently layer
1 mL	1% Cupric sulfate	Gently layer

4. Add 0.1 mL of each standard or sample to the corresponding tube and duplicates.

5. Add 1.0 mL of Solution A to each tube including blank. Vortex each tube after Solution A is added to all.
6. Incubate at room temperature for 10 min.
7. Dilute Folin & Ciocalteu's Phenol Reagent (Sigma F-9252) 1:2 with ddH_2O. Stir briefly.
8. Pipet 0.1 ml of diluted Folin & Ciocalteu's Phenol Reagent directly into the center of each tube while vortexing each tube without touching sides of tube.
13. Incubate 30 min at room temperature.
14. Turn on spectrophotometer at start of incubation. Select setting a:/Low_Pro at 750 nm.
15. After incubation, read absorbance of all standards and samples in duplicate at 750 nm.

CALCULATIONS:

1. Generate a standard curve from absorbances of the 6 standards.
2. Using the equation from the standard curve, calculate the concentration of each sample.
3. Multiply the calculated concentration by the dilution factor (30) for the final protein concentration of each sample.

Appendix I: SDS-PAGE Procedure

(Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis)
(Shapiro et al., 1967)

SOLUTIONS:

30% acrylamide and 1% bisacrylamide mixture (200 ml):
58 g acrylamide (Sigma A-8887)
2 g bisacrylamide (Sigma M-7279)
Add reagents into 150 ml ddH_2O in the order listed, stir on a magnetic plate and bring final volume to 200 ml with ddH_2O.
Filter mixture through Whatman No. 1 filter paper. Store in a dark bottle at 4^oC.

1.5 M Tris, pH 8.8 (500 ml):
Add 90.82 g Trisbase (Sigma T-6066) into 300 ml ddH_2O, pH to 8.8 with concentrated HCl, stir on a magnetic plate and bring final volume to 500 ml with ddH_2O. Store at 4^oC.

1.0 M Tris, pH 6.8 (500 ml):
Add 60.57 g Trisbase (Sigma T-6066) into 300 ml ddH_2O, pH to 6.8 with concentrated HCl, stir on a magnetic plate and bring final volume to 500 ml with ddH_2O. Store at 4^oC.

10% Sodium dodecyl sulfate (SDS) (100 ml):
Add 10 g SDS (Sigma L-3771) into 80 ml ddH_2O, stir on a magnetic plate and bring final volume to 100 mL with ddH_2O. Filter through Whatman No. 1 filter paper. Store at room temperature.

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20% Sodium dodecyl sulfate (SDS) (100 ml):
Add 20 g SDS (Sigma L-3771) into 80 ml ddH_2O, stir on a magnetic plate and bring final volume to 100 mL with ddH_2O. Filter through Whatman No. 1 filter paper. Store at room temperature.

10% Ammonium persulfate (APS) (1 ml):
Add 100 mg APS (Sigma A-3678) into 1 ml ddH_2O, vortex until dissolve. Prepare fresh daily.

TEMED (N,N,N',N'-Tetramethylethylenediamine)
Sigma T-9281. Store at 4^oC.

10x Running buffer (1 L):
30.28 g Trisbase (Sigma T-6066)
144.2 g Glycine (Sigma G-8898)
Add reagents into 800 ml ddH_2O, stir on a magnetic plate and bring final volume to 1000 ml with ddH_2O.

1x Running buffer (1 L):
100 ml 10x Running buffer
5 ml 20% SDS
900 ml ddH_2O

0.25% Bromophenol blue (5 ml):
Add 0.0125 g bromophenol blue (Sigma B-5525) into 5 ml ddH_2O, stir on a magnetic plate.

2x Sample buffer (50 ml):
5 ml 1.25 M Tris, pH 6.8
10 ml 20% glycerol
5 ml 20% SDS
2.25 ml β-mercaptoethanol (Sigma M-6250) (add under fume hood)
1.6 ml 0.25% bromophenol blue solution (add 0,0125g into 5ml ddH2O to make 0.25%)
Add reagents to 26.15 ml ddH_2O, stir on a magnetic plate.

Resolving Gel (10%)

	For 2 gels (ml)	For 4 gels (ml)
ddH_2O	5.9	11.8
30% Acrylamide mix	5.0	10.0
1.5 M Tris, pH 8.8	3.8	7.6
10% SDS	0.15	0.3
10% APS (make fresh)	0.15	0.3
TEMED	0.006	0.012

Stacking Gel (5%)

	For 2 gels (ml)	For 4 gels (ml)
ddH_2O	3.4	6.8
30% Acrylamide mix	0.83	1.7
1.0 M Tris, pH 6.8	0.63	1.25
10% SDS	0.05	0.1
10% APS (make fresh)	0.05	0.1
TEMED	0.005	0.01

PROCEDURE:

1. Assemble gel apparatus in casting stands according to manufacturer's instructions.
2. Prepare resolving gel solution (10%) for the number of gels to be cast.
3. Gently swirl solution to avoid bubbles.
4. Use 1ml pipette to fill the caster ~3/4 full. Overlay resolving gel with 200 µl butanol to amooth out the top surface of the resolving gel.
5. Allow 1 hr for gel to polymerize.
6. Wash butanol off the resolving gel with ddH_2O. Dry between casting plates as much as possible with a KimWipe.

7. Prepare stacking gel solution for the number of gels to be cast.
8. Gently swirl solution. Use pipette to fill up the rest of the caster to the top with the stacking gel solution. Carefully insert 15-well comb into place, avoiding any bubble formation.
9. Allow ~45 min for stacking gel to polymerize.
10. During stacking gel polymerization, prepare samples with 2x sample buffer.
    a. Thaw samples on ice.
    b. Dilute the samples 1:1 with 2x sample buffer in 0.5 ml microcentrifuge tubes.
    c. Vortex tubes then place them in a tube holder in boiling water (~95°C) for 5 min.
    d. Centrifuge tubes in microcentrifuge for ~5 seconds to allow all solution collect in the bottom of the microcentrifuge tube.
11. After stacking gels have polymerized, carefully remove the 15-well combs and assemble the gel apparatus and electrophoresis chamber according to the manufacturer's instructions. Fill inner chamber with 1x Running buffer and the outer chamber to ~1/3 full with 1x Running buffer (~500 ml for each chamber).
12. Load 100 μg muscle homogenate (~10 – 20 μl sample + buffer), rat muscle standard and moleculare weight marker into gel wells.
13. Electrophoresis at 160 V for 2hr, may extend for 10-20 more min, make sure the 50 kDa band not running out.

Appendix J: Western Blotting Procedures

(Wet Transfer)

SOLUTIONS:

Transfer Buffer (2 L):
1400 ml ddH2O
28.8 g Glycine (Sigma G-8898)
6.04 g Trisbase (Sigma T-6066)
0.75 g SDS (Sigma L-3771) (or use 3.75 ml 20% SDS)
300 m Methanol (Fisher A-411-4)

Bring volume to 2000ml with ddH2O, stir on a magnetic plate.

PROCEDURE:

1. Soak filter papers (2 for each gel) in transfer buffer for 20 min.
2. Prepare nitrocellulose membrane, cut to ~6 cm x 9.5 cm each, mark in pencils in the left edge for identification
3. Soak nitrocellulose membranes in transfer buffer on a shake for 15 min.
4. Carefully remove the gel from the electrophoresis apparatus. Make cuts in the gel corners for identification. Place gel in transfer buffer on a shaker for 15 min.
5. Soak four spongy pads in transfer buffer for a few minutes
6. Making the gel "sandwich":
    a. Place cassette in a container filled with transfer buffer. Black side down, clear side up and facing away from you.
    b. Place a spongy pad on the surface of black side
    c. Place a filter paper on the top of spongy pad
    d. Place gel on the top of the filter paper. Use finger to direct the gel and let it lie on the middle surface of filter paper
    e. Place nitrocellulose membrane to cover the gel. Use the fingertip to touch down the membrane slightly and let it fully contact with the gel.
    f. Place another filter paper on the top of the membrane. Roll out bubbles with wetted glass tube.
    g. Add another spongy pad on the top of the filter paper
    h. Close cassette. Make sure everything is aligned correctly.
    i. Place the cassette in transfer apparatus with the black sides lining up.
    j. Repeat steps a-i with the second gel

k. Place an ice cube in transfer apparatus

7. Loading the transfer apparatus and start the transfer
    a. Place the transfer apparatus in a large plastic container and fill the container with ice water to cover half of the transfer apparatus
    b. Fill the transfer apparatus with transfer buffer up to the edge.
    c. Place the top cover with electrodes to the apparatus
    d. Set voltage to 90 volts. Start and run for 2 hrs.

8. After transfer, turn off the voltage
    a. Take out the "sandwich" and place black side up.
    b. Remove spongy filter, filter paper.
    c. Carefully remove the gel and place it in container with some ddH_2O, put on a shaker for 2 min

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Appendix K: Immunoblotting Procedures

SOLUTIONS:

10 x Tris-buffered saline (TBS; pH 7.5) (1 L):
60.05 g Trisbase (Sigma T-6066)
87.6 g NaCl (Fisher S271-3)

ADD reagents into 800 ml ddH_2O, stir on a magnetic plate, pH to 7.5 with concentrated HCl, bring final volume to 1000 ml with ddH_2O.

1x Tris-buffered saline + 0.06% Tween-20 (1 xTTBS) (1 L):
100 ml 10 x Tris-buffered saline (TBS; pH 7.5)
900 ml ddH_2O
0.6 ml Tween-20 (Sigma P-1379)

Blocking solution (30 ml for each membrane):
7% Non-fat dry milk (NFDM) in 1x TTBS
Stir gently on a magnetic plate

Primary Antibody (diluted in 2% NFDM-TTBS):

	Dilution ratio	Probe site	Catalog #
p-mTOR	1:900	Ser-2448	Cell Signaling, 2971S
p-AS160	1:800	Thr-642	Cell Signaling, 4288S
p-GS	1:1000	Ser-641	Cell Signaling, 3891S
p-Akt/PKB	1:1000	Ser-473	Cell Signaling, 9271S
α-Tubulin	1:1000	Total	Cell Signaling, 2144S

Secondary Antibody:
Goat Anti-rabbit IgG (Cell Signaling 7074), diluted to 1:800 in 2% NFDM-TTBS

Stripping buffer, pH 6.7 (1 L):
20 g 2% sodium dodecyl sulphate (SDS) (Sigma L-3771)
7.5 g Trisbase (Sigma T-6066)
Add reagents into 850 ml ddH2O, stir on a magnetic plate
Add 7 ml of 100 mM 2-mercaptoethanol (perform this step under fume hood)
Adjust pH to 6.7
Bring final volume to 1000 ml with ddH2O

PROCEDURE:

1. Block nitrocellulose membrane in 7% blocking solution (above) for 30 min at room temperature with gentle shaking.
2. Wash membrane in ddH2O for 2 min

3. Wash membrane in 30 ml TTBS 3X for 5 min each on a shaker
4. Cut nitrocellulose membranes in sections according to molecular weight markers to probe individually for proteins of interest.
5. Incubate each membrane section in 10 ml of the respective primary antibody solutions overnight on a rocking platform at low speed (1.5) in 4ºC refrigerator.
6. Next morning, recycle the primary antibody and keep in the -20ºC refrigerator
7. Wash membrane in 15 ml TTBS 3X for 5 min each on a shaker
8. Incubate each membrane section in 10 ml of secondary antibody solutions for 1 hr with gentle shaking at room temperature.
9. Recycle the primary antibody and keep in the -20ºC refrigerator
10. Wash membrane in 15 ml TTBS 3X for 5 min each on a shaker
11. Visualize bands using ECL detection (ECL Western Lighting Plus kit, Perkin Elmer) with a Bio-Rad ChemiDoc detection system according to the manufacturer's instructions.
12. Analyze bands (intensity x mm^2) using Quantity One Analysis software.
13. Wash membrane sections in 15 ml TTBS for 5 min on a shaker
14. Dry membranes with thick transfer papers and store between two transfer papers in in 4ºC.
15. If stripping to reprobe, wash membranes in 15 ml TTBS 2X for 5 min each on a shaker
16. Incubate membranes in 30 ml stripping buffer for 1 hr in 70ºC shaking water bath at 65 rpm.
17. Wash membranes in 15 ml TTBS 2X for 5 min on a shaker
18. Start procedures with blocking step and follow procedures forward.

Appendix L: Raw Data

Study 1

Study 2

Study 1		Subject Characteristics						
Subject #	**Initials**	**Gender**	**Age (yr)**	**Weight (kg)**	**Height (cm)**	**BMI (kg/m^2)**	**Waist (cm)**	**Screen Fasting Blood Glucose (mmol/L)**
1	ADO	F	22	92.99	158.5	36.7	107	5.88
2	LWD	F	38	80.40	162.6	30.4	94	6.16
3	PGD	M	24	116.57	180.4	35.8	108	5.74
4	SDG	F	39	89.24	163.4	33.4	99.5	6.38
5	JHS	F	23	103.65	169.4	36.1	122.5	5.16
6	G-A	F	22	87.66	163.1	33	103	5.55
7	EAS	F	41	72.67	169.1	25.4	90	5.77
8	N-W	F	27	88.34	158.75	34.8	93	5.55
9	K-C	F	24	83.69	150.1	37.1	99	5.63
10	BCW	M	23	99.00	182.1	29.8	104	4.16
11	DLM	M	28	94.35	180.1	29	105	4.86
12	JLP	F	37	89.02	160.8	34.4	96	4.88
13	MLL	F	35	113.17	148	51.6	121	4.91
14	YMA	F	29	96.50	157.6	38.8	114	6.27
15	M-C	F	40	106.14	182.5	31.8	102	5.22
16	ANE	F	21	96.30	161.7	36.8	118	4.52
17	AHC	F	40	83.14	162.2	31.6	103	5.16
18	MMW1	F	27	99.79	165.1	36.6	112	5.99
19	MMW2	F	35	94.80	164.4	33.5	119	5.16
20	TDT	F	43	67.81	140.5	34.3	99	5.52
21	M-L	F	36	80.97	158.3	32.3	109	4.80
22	CRT	F	25	103.19	166.6	37.2	105	6.27
AVG			**30.86**	**92.70**	**163.88**	**34.56**	**105.59**	**5.44**
SE			**1.60**	**2.58**	**2.29**	**1.06**	**1.96**	**0.13**

Study 1		Plasma Glucose Concentration (mmol/L)					
		CHO					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	4.56	5.25	6.89	5.89	5.64	4.65
2	LWD	4.12	5.61	5.60	3.67	3.45	4.18
3	PGD	3.84	6.29	6.78	4.98	4.10	3.88
4	SDG	4.21	4.70	5.00	5.24	4.58	4.21
5	JHS	4.12	4.81	5.94	4.71	4.03	3.55
6	G-A	4.24	4.66	6.49	6.94	6.13	4.90
7	EAS	4.18	4.87	5.28	4.52	2.88	3.33
8	N-W	4.01	5.98	6.55	5.06	3.98	3.97
9	K-C	4.61	6.21	8.11	8.04	5.53	5.51
10	BCW	4.02	5.38	6.39	4.37	4.32	3.09
11	DLM	3.55	5.06	5.97	4.10	3.47	3.42
12	JLP	4.09	5.62	6.86	4.87	3.22	3.64
13	MLL	4.46	4.63	5.91	6.93	7.05	5.57
14	YMA	4.63	5.14	6.69	8.59	6.55	4.99
15	M-C	4.33	5.05	5.37	6.46	5.67	4.74
16	ANE	3.65	4.37	5.06	4.34	4.21	3.83
17	AHC	4.20	4.70	5.57	6.22	5.89	4.87
18	MMW	3.81	4.38	5.43	5.96	3.77	3.57
19	MMW2	4.56	5.29	7.77	9.70	7.88	5.39
20	TDT	4.13	5.55	7.58	5.07	4.08	4.86
21	M-L	4.03	4.89	6.91	9.10	7.84	5.16
22	CRT	4.28	5.30	6.38	6.13	3.40	4.63
AVG		**4.16**	**5.17**	**6.30**	**5.95**	**4.89**	**4.36**
SE		**0.06**	**0.12**	**0.19**	**0.36**	**0.32**	**0.16**
		CHO/AA					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	4.04	4.55	5.39	5.62	5.24	4.95
2	LWD	4.13	5.10	4.63	2.76	2.45	2.79
3	PGD	4.48	6.16	7.29	5.75	3.80	3.50
4	SDG	4.41	4.29	4.39	3.72	3.32	3.70
5	JHS	4.07	6.61	4.50	3.16	4.10	3.68
6	G-A	4.08	4.36	5.77	4.68	4.92	4.16
7	EAS	3.78	4.36	4.28	4.40	4.73	4.78
8	N-W	3.89	4.05	3.69	4.76	4.11	3.34
9	K-C	4.41	5.09	6.29	5.11	4.92	4.46
10	BCW	4.89	5.65	5.53	4.97	3.53	3.19
11	DLM	3.82	4.34	5.29	3.12	2.84	2.98
12	JLP	3.84	5.26	5.83	4.18	3.73	3.62
13	MLL	4.20	4.46	5.61	5.40	4.49	4.16
14	YMA	4.53	5.00	6.42	7.04	4.85	4.18
15	M-C	4.06	4.52	3.95	3.97	3.83	3.92
16	ANE	3.93	4.67	4.17	2.46	3.16	3.20
17	AHC	4.09	4.58	6.10	4.08	4.54	3.79
18	MMW	3.75	4.58	4.88	4.36	3.52	2.87
19	MMW2	4.79	5.84	7.54	8.30	6.32	4.19
20	TDT	4.32	5.25	5.65	3.51	3.93	3.89
21	M-L	3.87	5.00	5.58	5.41	6.15	5.72
22	CRT	4.62	4.95	5.74	6.78	4.05	4.07
AVG		**4.18**	**4.94**	**5.39**	**4.71**	**4.21**	**3.87**
SE		**0.07**	**0.14**	**0.22**	**0.31**	**0.21**	**0.15**

Study 1		Plasma Insulin Concentration (pmol/L)					
		CHO					
Subject #	Initials	Pre	15min	30min	60min	120min	180min
1	ADO	248.28	707.00	2010.37	2677.99	1823.76	885.97
2	LWD	114.45	475.11	529.83	478.86	255.30	287.38
3	PGD	101.19	793.05	1210.17	538.17	181.33	125.36
4	SDG	92.30	239.39	297.94	474.76	305.16	300.02
5	JHS	174.46	752.07	1828.06	1474.08	611.37	405.80
6	G-A	132.51	559.14	1313.37	1753.68	1493.59	1011.54
7	EAS	115.91	259.88	501.36	799.23	381.56	292.87
8	N-W	171.96	809.86	1739.58	1810.91	1129.12	731.10
9	K-C	242.17	830.83	1557.62	1489.08	1213.22	853.33
10	BCW	92.09	421.08	539.56	731.66	317.46	103.97
11	DLM	140.22	672.28	1553.04	951.19	233.42	339.12
12	JLP	93.06	601.85	1193.29	982.86	469.76	356.90
13	MLL	330.44	601.78	1176.76	1173.01	2278.86	1483.45
14	YMA	214.46	418.92	305.51	1273.78	2289.21	1157.66
15	M-C	218.14	446.08	977.23	1575.06	1383.58	1039.04
16	ANE	137.02	734.29	1983.63	1445.12	1082.24	725.68
17	AHC	112.93	620.33	656.65	889.65	792.49	454.76
18	MMW	173.90	337.04	664.57	1293.09	1007.79	511.01
19	MMW2	180.57	334.19	1052.65	1132.52	795.55	540.46
20	TDT	85.98	645.75	1312.12	804.02	440.24	130.36
21	M-L	286.97	662.34	704.78	2098.57	3253.04	1893.00
22	CRT	144.59	249.95	292.32	491.01	149.73	238.91
AVG		**163.80**	**553.28**	**1063.66**	**1197.19**	**994.90**	**630.35**
SE		**14.53**	**40.68**	**118.94**	**121.13**	**175.37**	**99.63**
		CHO/AA					
Subject #	Initials	Pre	15min	30min	60min	120min	180min
1	ADO	234.67	607.90	1783.48	2538.12	2270.11	1402.20
2	LWD	122.93	590.19	964.24	328.64	233.00	238.21
3	PGD	100.91	511.36	1193.71	797.70	286.55	132.16
4	SDG	128.41	322.11	465.04	503.03	276.41	271.13
5	JHS	170.92	255.02	2706.26	1037.79	1838.48	923.27
6	G-A	232.80	479.14	1407.82	1155.58	1103.98	524.07
7	EAS	71.46	270.99	326.55	321.76	637.76	419.06
8	N-W	120.84	449.90	1125.51	1343.86	753.67	443.99
9	K-C	290.51	1568.88	2758.00	1595.06	1825.08	2078.85
10	BCW	146.12	737.21	1582.00	466.08	406.98	133.48
11	DLM	170.85	421.28	946.40	1364.97	473.93	640.88
12	JLP	115.63	552.06	1253.36	1396.92	616.51	467.26
13	MLL	261.97	708.46	1819.17	1442.62	1048.90	1435.60
14	YMA	266.34	526.36	721.79	1916.82	1971.69	665.61
15	M-C	201.47	1607.49	1973.42	1080.02	805.20	461.56
16	ANE	109.24	1147.31	2552.98	787.29	604.35	248.63
17	AHC	104.80	596.30	697.28	610.33	768.95	374.34
18	MMW	144.04	332.18	778.26	831.11	959.38	596.30
19	MMW2	134.18	388.78	840.14	1057.65	1898.97	677.83
20	TDT	102.58	632.27	1322.88	607.76	424.48	228.49
21	M-L	220.43	711.86	1385.32	1341.64	1981.13	2665.21
22	CRT	98.41	275.99	175.36	1032.51	494.41	360.51
AVG		**161.34**	**622.41**	**1308.13**	**1070.78**	**985.45**	**699.48**
SE		**13.66**	**79.19**	**155.00**	**114.64**	**140.69**	**138.31**

Study 1		Plasma C-peptide Concentration (nmol/L)					
		CHO					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	1.31	2.10	3.65	4.23	3.92	2.95
2	LWD	0.61	1.48	1.75	2.12	1.12	1.48
3	PGD	0.40	1.75	1.98	1.70	0.90	0.56
4	SDG	0.48	0.81	1.09	1.82	1.83	1.49
5	JHS	0.65	1.52	3.22	3.18	2.33	1.81
6	G-A	0.64	1.03	2.40	3.20	3.20	2.68
7	EAS	0.42	0.78	1.31	2.17	1.42	1.47
8	N-W	0.81	1.74	2.69	3.03	2.61	2.00
9	K-C	0.76	1.43	2.00	2.42	2.21	1.79
10	BCW	0.20	0.74	1.42	1.99	1.28	0.31
11	DLM	0.41	1.44	2.89	2.15	1.78	1.10
12	JLP	0.31	1.19	1.69	1.85	1.19	1.02
13	MLL	1.07	1.61	2.45	2.55	3.35	2.88
14	YMA	0.93	1.19	1.59	2.78	3.22	2.54
15	M-C	0.74	1.13	2.02	3.19	2.89	2.27
16	ANE	0.65	1.28	3.13	2.96	2.58	2.03
17	AHC	0.75	1.62	2.38	3.21	3.04	2.40
18	MMW	0.68	1.00	1.66	2.97	2.63	1.73
19	MMW2	0.79	1.10	2.30	2.66	2.76	2.44
20	TDT	0.43	1.61	2.72	2.97	2.24	1.36
21	M-L	0.80	1.35	2.42	3.08	3.51	3.04
22	CRT	0.74	0.91	1.48	2.17	1.21	1.57
AVG		**0.66**	**1.31**	**2.19**	**2.66**	**2.33**	**1.86**
SE		**0.05**	**0.08**	**0.14**	**0.13**	**0.19**	**0.16**
		CHO/AA					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	1.05	1.83	3.12	4.16	3.99	3.87
2	LWD	0.80	1.82	2.80	2.18	1.74	1.42
3	PGD	0.48	1.32	2.12	2.28	1.19	0.66
4	SDG	0.54	1.01	1.47	1.91	1.34	1.36
5	JHS	0.67	0.58	3.88	2.81	3.57	2.43
6	G-A	0.84	1.21	2.68	2.89	2.85	1.93
7	EAS	0.36	0.77	0.95	1.01	2.16	1.84
8	N-W	0.58	1.07	2.28	3.04	2.50	1.94
9	K-C	0.87	2.12	3.18	2.63	3.02	3.15
10	BCW	0.43	1.42	2.31	1.64	1.73	0.50
11	DLM	1.00	1.18	2.01	2.89	1.85	2.23
12	JLP	0.32	0.99	1.97	2.64	1.52	1.20
13	MLL	1.06	1.75	2.75	3.07	2.94	3.01
14	YMA	0.95	1.35	2.04	3.11	3.60	2.51
15	M-C	0.62	2.29	3.08	2.55	2.21	1.90
16	ANE	0.59	1.96	3.59	2.26	2.09	1.22
17	AHC	0.63	1.50	1.36	2.43	3.29	2.40
18	MMW	0.59	0.99	1.85	2.43	2.43	2.27
19	MMW2	0.77	1.15	1.85	2.48	3.96	2.88
20	TDT	0.47	1.29	2.72	2.44	2.13	1.39
21	M-L	0.78	1.70	2.43	2.43	3.22	3.76
22	CRT	0.60	0.95	1.44	2.78	2.30	1.59
AVG		**0.68**	**1.38**	**2.36**	**2.55**	**2.53**	**2.07**
SE		**0.05**	**0.10**	**0.16**	**0.13**	**0.18**	**0.19**

Study 1		Plasma Glucagon Concentration (ng/L)					
		CHO					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	47.25	54.00	35.69	40.38	43.28	45.47
2	LWD	39.80	34.40	25.90	24.29	29.31	24.05
3	PGD	73.33	51.24	46.97	53.42	48.69	54.28
4	SDG	69.16	71.07	68.72	84.03	71.73	71.68
5	JHS	122.45	104.77	111.50	93.26	86.91	96.87
6	G-A	43.01	60.87	63.15	44.06	40.25	35.09
7	EAS	30.95	33.65	28.90	42.28	41.80	43.62
8	N-W	69.72	68.03	67.66	58.61	54.64	57.85
9	K-C	86.69	56.72	42.86	33.30	47.28	46.24
10	BCW	34.21	46.25	39.31	34.66	33.74	47.51
11	DLM	52.88	41.33	40.50	51.19	43.04	42.68
12	JLP	51.45	60.17	50.99	48.83	55.35	49.48
13	MLL	64.10	51.14	41.28	58.67	60.00	48.50
14	YMA	148.97	138.64	110.04	110.52	112.95	109.88
15	M-C	46.03	53.50	47.75	39.94	35.92	38.03
16	ANE	73.09	41.94	42.80	42.05	36.28	55.84
17	AHC	54.71	47.71	38.77	45.82	46.63	48.93
18	MMW	87.40	68.50	66.77	59.49	64.47	61.77
19	MMW2	63.49	66.74	66.37	52.12	52.45	29.87
20	TDT	122.76	130.27	134.25	120.94	117.96	134.31
21	M-L	40.74	34.99	24.79	31.83	28.55	32.68
22	CRT	67.23	52.49	39.14	48.45	72.56	67.53
AVG		**67.70**	**62.20**	**56.10**	**55.37**	**55.63**	**56.46**
SE		**6.51**	**6.00**	**6.17**	**5.35**	**5.18**	**5.68**
		CHO/AA					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	58.53	58.10	41.29	37.40	24.94	36.42
2	LWD	35.04	35.40	39.64	34.84	37.42	33.39
3	PGD	69.69	57.12	49.08	53.19	51.65	62.70
4	SDG	70.67	58.00	63.60	80.18	84.79	80.28
5	JHS	98.22	97.00	102.63	92.49	92.32	100.64
6	G-A	58.32	55.64	50.40	38.23	34.26	26.17
7	EAS	48.19	33.76	35.00	62.89	51.66	53.83
8	N-W	66.22	56.55	59.38	60.08	61.41	66.96
9	K-C	68.37	63.34	47.57	41.65	49.32	49.80
10	BCW	47.63	49.66	39.10	42.69	43.81	55.15
11	DLM	44.14	47.80	38.90	45.07	45.20	44.72
12	JLP	44.22	50.82	55.82	70.56	54.34	54.28
13	MLL	54.05	69.96	61.03	51.15	38.21	44.57
14	YMA	146.84	153.84	146.86	152.32	139.51	136.97
15	M-C	44.65	58.56	54.00	47.08	42.96	36.78
16	ANE	40.28	29.64	44.68	67.01	51.59	58.90
17	AHC	54.23	49.50	40.57	51.94	53.81	45.52
18	MMW	66.66	80.61	80.48	81.62	78.53	64.01
19	MMW2	47.77	65.72	51.11	50.76	42.83	39.89
20	TDT	127.29	128.81	133.18	134.82	131.80	129.31
21	M-L	44.61	40.52	27.61	28.62	29.90	33.67
22	CRT	68.96	75.46	48.72	78.58	74.99	73.40
AVG		**63.84**	**64.35**	**59.58**	**63.78**	**59.78**	**60.33**
SE		**5.91**	**6.31**	**6.53**	**6.60**	**6.40**	**6.24**

Study 1		Plasma Lactate Concentration (mmol/L)					
		CHO					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	1.257	1.285	1.393	1.719	1.313	1.075
2	LWD	0.431	0.511	0.700	0.890	0.762	0.721
3	PGD	0.742	0.828	0.980	1.107	0.943	0.867
4	SDG	0.675	0.652	0.719	1.066	0.941	0.902
5	JHS	0.807	0.778	0.781	1.277	1.408	1.181
6	G-A	1.217	1.262	1.469	2.231	1.559	1.103
7	EAS	1.187	0.981	1.023	1.290	1.046	1.046
8	N-W	1.097	1.111	1.371	1.797	1.475	1.278
9	K-C	0.981	0.843	1.198	1.350	1.110	1.032
10	BCW	0.691	0.696	0.686	1.033	1.244	0.932
11	DLM	0.975	0.934	1.177	1.554	1.149	0.932
12	JLP	0.860	0.551	0.694	1.044	0.932	0.829
13	MLL	0.991	0.869	0.910	1.259	1.281	1.083
14	YMA	1.331	1.698	1.162	1.474	1.252	1.176
15	M-C	1.097	1.035	0.968	1.568	1.673	1.355
16	ANE	0.730	0.794	0.887	0.325	1.449	1.087
17	AHC	1.146	1.066	1.198	1.719	1.106	0.836
18	MMW	0.632	0.568	0.612	0.995	0.926	0.744
19	MMW2	1.051	0.892	1.166	1.494	1.084	0.799
20	TDT	0.513	0.559	0.781	1.167	0.865	0.668
21	M-L	1.166	0.904	1.022	1.001	1.419	0.904
22	CRT	0.500	0.512	0.526	0.840	0.810	0.717
AVG		**0.913**	**0.879**	**0.974**	**1.282**	**1.170**	**0.967**
SE		**0.057**	**0.062**	**0.057**	**0.086**	**0.055**	**0.040**
		CHO/AA					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	1.140	1.167	1.293	1.420	1.056	0.953
2	LWD	1.139	0.711	1.140	1.207	0.819	0.829
3	PGD	1.031	0.932	1.002	1.213	1.045	0.672
4	SDG	0.991	0.902	0.907	1.096	1.115	1.186
5	JHS	1.067	0.889	1.186	1.316	1.530	1.565
6	G-A	1.149	1.019	1.240	1.531	1.276	1.084
7	EAS	1.095	0.967	0.921	1.275	1.052	1.108
8	N-W	1.362	1.238	1.406	1.781	1.569	1.131
9	K-C	0.921	0.926	1.120	1.414	1.159	1.091
10	BCW	0.886	0.978	1.308	1.589	1.184	0.973
11	DLM	1.211	1.378	1.021	1.225	1.050	0.914
12	JLP	1.254	0.644	0.735	1.193	1.216	1.117
13	MLL	0.704	0.836	0.905	1.432	1.124	1.100
14	YMA	0.945	0.906	0.921	1.466	1.266	1.018
15	M-C	0.999	0.897	1.283	1.484	1.547	1.586
16	ANE	0.877	0.898	1.008	1.365	1.351	0.674
17	AHC	0.783	0.877	0.915	1.276	1.136	0.921
18	MMW	0.712	0.531	0.612	1.198	1.060	0.944
19	MMW2	0.806	0.856	0.747	1.424	1.130	1.044
20	TDT	0.778	0.796	1.072	1.385	1.041	0.870
21	M-L	0.768	0.610	0.723	1.055	0.999	0.912
22	CRT	0.503	0.605	0.503	1.083	0.975	0.735
AVG		**0.960**	**0.889**	**0.999**	**1.338**	**1.168**	**1.019**
SE		**0.045**	**0.043**	**0.051**	**0.038**	**0.041**	**0.049**

Study 1		Plasma Free Fatty Acids Concentration (mmol/L)					
		CHO					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	0.2853	0.2793	0.2543	0.0942	0.1089	0.0536
2	LWD	0.1555	0.1278	0.1239	0.0597	0.0589	0.0600
3	PGD	0.2353	0.2266	0.1739	0.1494	0.0992	0.1652
4	SDG	0.2112	0.2347	0.1946	0.0646	0.0226	0.0764
5	JHS	0.2527	0.2400	0.1527	0.0475	0.0356	0.0296
6	G-A	0.3919	0.2664	0.2402	0.1123	0.0825	0.0579
7	EAS	0.3357	0.2115	0.2243	0.1263	0.1257	0.0747
8	N-W	0.2240	0.2367	0.1464	0.0634	0.0829	0.0443
9	K-C	0.2064	0.2064	0.1560	0.0335	0.0467	0.0514
10	BCW	0.2685	0.2976	0.2206	0.0800	0.1380	0.0989
11	DLM	0.3246	0.2322	0.1725	0.1461	0.0879	0.1066
12	JLP	0.3943	0.3881	0.2020	0.0702	0.0799	0.0962
13	MLL	0.2498	0.1448	0.1510	0.0883	0.0660	0.0803
14	YMA	0.2237	0.3723	0.2519	0.0991	0.0222	0.0156
15	M-C	0.2935	0.5042	0.3274	0.1002	0.0356	0.0306
16	ANE	0.1850	0.2320	0.1514	0.0536	0.0099	0.0139
17	AHC	0.3091	0.3610	0.2283	0.1120	0.0658	0.0765
18	MMW	0.1779	0.1188	0.1022	0.0652	0.0961	0.0710
19	MMW2	0.2289	0.2394	0.2281	0.1013	0.0862	0.0566
20	TDT	0.2606	0.2105	0.1181	0.0569	0.0273	0.0097
21	M-L	0.2393	0.3084	0.1978	0.1201	0.1307	0.1174
22	CRT	0.2070	0.2075	0.1107	0.0300	0.0190	0.0274
AVG		**0.2573**	**0.2566**	**0.1876**	**0.0852**	**0.0694**	**0.0643**
SE		**0.0136**	**0.0191**	**0.0120**	**0.0072**	**0.0082**	**0.0081**
		CHO/AA					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	0.2447	0.2241	0.2054	0.1214	0.1284	0.0909
2	LWD	0.1681	0.1278	0.1183	0.0796	0.0804	0.0714
3	PGD	0.2570	0.1857	0.2034	0.1284	0.1110	0.1367
4	SDG	0.0964	0.1112	0.0762	0.0427	0.0468	0.0360
5	JHS	0.1614	0.0690	0.0773	0.0520	0.0623	0.0512
6	G-A	0.3105	0.1950	0.1746	0.1112	0.0853	0.0780
7	EAS	0.2707	0.3108	0.3331	0.3359	0.1557	0.1543
8	N-W	0.3092	0.2262	0.2381	0.0619	0.0601	0.0941
9	K-C	0.2710	0.1756	0.1489	0.0328	0.0287	0.0145
10	BCW	0.1022	0.0785	0.0545	0.0487	0.0655	0.0708
11	DLM	0.2003	0.1860	0.1570	0.0645	0.0661	0.1093
12	JLP	0.2794	0.2381	0.2266	0.0842	0.0546	0.0753
13	MLL	0.2549	0.2160	0.2146	0.0644	0.0369	0.0368
14	YMA	0.2661	0.2448	0.2914	0.0948	0.0205	0.0059
15	M-C	0.2224	0.1788	0.1222	0.0758	0.0296	0.0349
16	ANE	0.1501	0.1322	0.1399	0.1172	0.0250	0.0070
17	AHC	0.2653	0.2851	0.0592	0.0447	0.0217	0.0489
18	MMW	0.1611	0.0907	0.0849	0.0590	0.0527	0.0426
19	MMW2	0.3029	0.4165	0.3749	0.1729	0.1244	0.1350
20	TDT	0.2443	0.2080	0.1001	0.0559	0.0498	0.0489
21	M-L	0.2244	0.2316	0.2125	0.1332	0.1113	0.0769
22	CRT	0.1575	0.1771	0.0948	0.0712	0.0600	0.0624
AVG		**0.2236**	**0.1958**	**0.1685**	**0.0933**	**0.0671**	**0.0674**
SE		**0.0137**	**0.0171**	**0.0189**	**0.0138**	**0.0081**	**0.0087**

Study 1		Plasma Triglycerids Concentration (mmol/L)					
		CHO					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	1.639	1.495	1.470	1.558	1.637	1.430
2	LWD	0.586	0.734	0.758	0.635	0.579	0.360
3	PGD	0.976	0.860	0.699	0.817	0.625	0.667
4	SDG	0.700	0.660	0.732	0.763	0.770	0.651
5	JHS	0.413	0.472	0.396	0.566	0.539	0.491
6	G-A	1.102	1.072	1.458	1.415	0.994	0.958
7	EAS	0.408	0.389	0.391	0.471	0.436	0.343
8	N-W	0.957	0.951	1.164	1.140	0.884	0.816
9	K-C	1.434	1.315	1.362	1.674	1.433	1.168
10	BCW	0.618	0.584	0.756	0.671	0.503	0.430
11	DLM	0.880	1.071	1.319	1.117	1.317	1.677
12	JLP	0.688	0.654	0.641	0.817	0.703	0.651
13	MLL	0.285	0.552	0.541	0.715	0.480	0.490
14	YMA	1.703	1.711	1.740	1.806	1.916	2.051
15	M-C	0.646	0.738	0.809	0.747	0.546	0.567
16	ANE	0.574	0.601	0.685	0.821	0.654	0.534
17	AHC	1.994	2.046	1.979	1.959	1.684	1.943
18	MMW	0.864	1.015	0.902	0.977	0.858	0.686
19	MMW2	1.517	1.367	1.624	1.702	1.600	1.818
20	TDT	0.484	0.509	0.590	0.585	0.524	0.395
21	M-L	0.833	0.743	0.944	0.928	0.957	1.073
22	CRT	0.836	0.730	0.832	0.791	0.754	0.715
AVG		**0.915**	**0.921**	**0.991**	**1.031**	**0.927**	**0.905**
SE		**0.099**	**0.092**	**0.096**	**0.096**	**0.098**	**0.116**
		CHO/AA					
Subject #	**Initials**	**Pre**	**15min**	**30min**	**60min**	**120min**	**180min**
1	ADO	2.396	2.107	2.324	2.401	2.066	1.915
2	LWD	0.611	0.598	0.603	0.588	0.573	0.607
3	PGD	0.735	0.640	0.654	0.900	0.678	0.775
4	SDG	0.779	0.918	0.733	0.769	0.806	0.914
5	JHS	0.503	0.206	0.474	0.548	0.544	0.498
6	G-A	0.900	0.880	1.106	1.060	0.964	0.891
7	EAS	0.409	0.345	0.385	0.543	0.409	0.378
8	N-W	0.684	0.588	0.703	0.677	0.580	0.568
9	K-C	0.748	0.778	1.018	1.217	0.887	0.854
10	BCW	0.271	0.274	0.337	0.297	0.269	0.308
11	DLM	1.658	1.271	1.256	1.120	1.024	1.185
12	JLP	0.710	0.717	0.877	0.811	0.781	0.706
13	MLL	0.490	0.407	0.589	0.558	0.452	0.368
14	YMA	1.586	1.650	1.413	1.717	1.617	1.464
15	M-C	0.800	0.754	0.764	0.861	0.734	0.806
16	ANE	0.674	0.746	0.841	0.937	0.899	0.902
17	AHC	1.345	1.165	1.641	1.610	1.375	1.517
18	MMW	0.877	0.898	0.868	0.933	0.739	0.666
19	MMW2	1.283	1.335	1.143	1.237	1.415	1.635
20	TDT	0.681	0.567	0.595	0.633	0.681	0.648
21	M-L	0.835	0.791	1.229	0.946	0.918	0.947
22	CRT	0.924	0.648	0.807	0.884	0.666	0.682
AVG		**0.904**	**0.831**	**0.925**	**0.966**	**0.867**	**0.874**
SE		**0.103**	**0.096**	**0.097**	**0.100**	**0.090**	**0.091**

Study 2		Subject Characteristics								
Subject#	**Initials**	**Gender**	**Age**	**Height (cm)**	**Weight (kg)**	**VO2max (L/min)**	**VO2max (ml/kg/min)**	**Watts @ 70%VO2max**	**Watts @ 85%VO2max**	**Watts @ 45%VO2max**
001	JB	M	22	168	66.9	2.957	44.20	140	170	80
002	DGL	M	24	175	76.2	3.867	50.75	185	230	110
003	JSW	F	36	165	61.7	2.900	47.00	135	170	75
004	JLM	F	34	168	63.1	3.237	51.30	170	210	100
005	JWB	M	24	180	62.4	3.391	54.35	160	190	90
006	HMZ	F	20	178	71	2.900	40.85	140	175	80
007	BTI	M	21	192	82.2	4.410	53.65	220	270	130
008	ASD	M	26	182	87.3	4.540	52.00	225	280	140
009	JFC	M	37	169	77.4	3.878	50.10	190	240	115
010	BDK	M	31	175	68.1	3.957	58.10	195	235	110
AVG			**27.5**	**175.2**	**71.63**	**3.604**	**50.23**	**176**	**217**	**103**
SE			**2.03**	**2.59**	**2.80**	**0.194**	**1.60**	**10.30**	**12.81**	**6.96**

Study 2		Ratings of Perceived Exertion (RPE) and Heart Rate (HR, bpm)												
CHO														
Subject#	**Initials**	**RPE Ex_30**	**RPE Ex_60**	**RPE Ex_90**	**RPE Ex_120**	**RPE Sprints**	**RPE Wingate**	**HR Pre**	**HR Ex_30**	**HR Ex_60**	**HR Ex_90**	**HR Ex_120**	**HR Sprint**	**HR Wingate**
001	JB	15	17	17	18	20	19	56	150	147	150	158	170	167
002	DGL	13	14	14	17	20	19	53	156	154	154	156	171	178
003	JSW	13	13	14	14	19	20	59	147	158	156	158	183	213
004	JLM	12	12	12.5	13	17	17.5	57	135	134	134	140	174	175
005	JWB	12	12	13	13	19.5	19.5	61	135	130	130	143	174	169
006	HMZ	13	13	14	15	20	19	62	151	154	153	159	175	165
007	BTI	13	14	14	15	16	17.5	61	138	146	153	161	170	173
008	ASD	13	15	17	19	20	20	65	142	145	151	134	155	166
009	JFC	14	14	14	14	18	17.5	50	128	123	128	129	166	156
010	BDK	14	14	14	15	18	20	62	129	129	131	128	146	160
AVG		**13.20**	**13.80**	**14.35**	**15.30**	**18.75**	**18.90**	**58.60**	**141.10**	**142.00**	**144.00**	**146.60**	**168.40**	**172.20**
SE		**0.29**	**0.47**	**0.47**	**0.65**	**0.45**	**0.33**	**1.45**	**3.05**	**3.85**	**3.67**	**4.19**	**3.36**	**5.00**
CHO/LAA														
Subject#	**Initials**	**RPE Ex_30**	**RPE Ex_60**	**RPE Ex_90**	**RPE Ex_120**	**RPE Sprints**	**RPE Wingate**	**HR Pre**	**HR Ex_30**	**HR Ex_60**	**HR Ex_90**	**HR Ex_120**	**HR Sprint**	**HR Wingate**
001	JB	14	16	17	19	20	17	68	151	150	153	130	162	166
002	DGL	13	13	14	16	19	19	50	144	146	155	145	170	179
003	JSW	12.5	12	13	13	19	17	55	140	142	144	158	183	164
004	JLM	13	13	13.5	14	18.5	17	61	141	150	143	146	183	182
005	JWB	12	12	13	14	19	19.5	51	128	129	130	137	174	174
006	HMZ	13	13	14	14	19	19.5	59	141	141	145	149	174	164
007	BTI	13	14	14	15	17	15	71	137	145	148	153	171	172
008	ASD	13	14	15	16	20	20	70	133	136	139	143	173	164
009	JFC	14.5	15	15	15.5	17	18.5	80	138	142	145	146	162	155
010	BDK	13	14	15	15	17	17	59	133	131	137	132	147	164
AVG		**13.10**	**13.60**	**14.35**	**15.15**	**18.55**	**17.95**	**62.40**	**138.60**	**141.20**	**143.90**	**143.90**	**169.90**	**168.40**
SE		**0.22**	**0.40**	**0.38**	**0.53**	**0.37**	**0.50**	**3.05**	**2.04**	**2.29**	**2.34**	**2.79**	**3.39**	**2.59**

Study 2		Ratings of Perceived Exertion (RPE) and Heart Rate (HR, bpm)												
		CHO/HAA												
Subject#	**Initials**	**RPE Ex_30**	**RPE Ex_60**	**RPE Ex_90**	**RPE Ex_120**	**RPE Sprints**	**RPE Wingate**	**HR Pre**	**HR Ex_30**	**HR Ex_60**	**HR Ex_90**	**HR Ex_120**	**HR Sprint**	**HR Wingate**
001	JB	14	16	16	17	19	18	61	148	148	155	160	177	166
002	DGL	13	13	14	16	19	19	60	149	157	148	153	175	180
003	JSW	12.5	13	14.5	15	20	19.5	51	146	159	164	176	181	187
004	JLM	12.5	12.5	13	13	17	17.5	63	132	138	144	141	174	184
005	JWB	12	13	13	15	19	19.5	68	139	135	141	140	166	178
006	HMZ	13	14	13	13	19	19	69	149	150	144	151	182	175
007	BTI	13	13	14	14	16	17	83	147	155	151	166	182	171
008	ASD	15	16	17	17	20	20	101	151	151	151	148	170	164
009	JFC	13.5	15	16	17	17	17	51	143	135	153	156	173	156
010	BDK	13	14	15	14	17	20	67	122	125	125	128	173	173
AVG		**13.15**	**13.95**	**14.55**	**15.10**	**18.30**	**18.65**	**67.40**	**142.60**	**145.30**	**147.60**	**151.90**	**175.30**	**173.40**
SE		**0.27**	**0.41**	**0.45**	**0.50**	**0.45**	**0.37**	**4.75**	**2.91**	**3.59**	**3.26**	**4.36**	**1.67**	**3.01**

Study 2		Wingate Anaerobic Test											
CHO													
Subject#	**Initials**	**Mean Watts**	**Peak Watts**	**Min. Watts**	**Peak Watts reached @ (sec)**	**Mean RPM**	**Peak RPM**	**Min. RPM**	**Peak RPM reached @ (sec)**	**Anaerobic Capacity (w/kg)**	**Anaerobic Power (w/kg)**	**Fatigue Index (w/s)**	**Total Work (joules)**
001	JB	505	672	389	0.1	124	153	112	0.4	7.6	10.1	9.5	15140.2
002	DGL	618	986	418	0.1	136	171	99	1.7	8.1	13	19	18536.3
003	JSW	468	738	320	0.1	136	166	103	1.3	7.8	12.3	14	14029.3
004	JLM	517	725	404	0.1	140	154	119	4.5	8.4	11.7	10.8	15512.6
005	JWB	523	839	350	0.1	142	173	108	1.4	8.5	13.6	16.4	15682.5
006	HMZ	465	796	323	0.1	115	156	86	0.5	6.6	11.3	15.8	13954
007	BTI	667	768	536	0.1	129	149	116	0.2	8	9.3	7.8	20015.5
008	ASD	656	1114	394	0.1	127	171	93	1.1	7.5	12.8	24.1	19686.7
009	JFC	556	936	405	0.1	122	166	93	0.6	7.2	12.1	17.8	16692.7
010	BDK	562	890	399	0.1	139	177	108	1.1	8.3	13.1	16.4	16868.5
AVG		**553.70**	**846.40**	**393.80**	**0.10**	**131.00**	**163.60**	**103.70**	**1.28**	**7.80**	**11.93**	**15.16**	**16611.83**
SE		**22.95**	**43.03**	**19.28**	**0.00**	**2.83**	**3.10**	**3.43**	**0.39**	**0.19**	**0.43**	**1.53**	**689.50**
CHO/LAA													
Subject#	**Initials**	**Mean Watts**	**Peak Watts**	**Min. Watts**	**Peak Watts reached @ (sec)**	**Mean RPM**	**Peak RPM**	**Min. RPM**	**Peak RPM reached @ (sec)**	**Anaerobic Capacity (w/kg)**	**Anaerobic Power (w/kg)**	**Fatigue Index (w/s)**	**Total Work (joules)**
001	JB	546	691	422	0.1	133	153	114	0.5	8.2	10.4	9	16384.1
002	DGL	618	933	449	0.1	135	168	105	0.7	8.1	12.3	16.2	18536.2
003	JSW	443	668	311	0.1	126	145	100	2.1	7.4	11.2	12	13281.7
004	JLM	508	628	395	0.1	134	143	119	9.6	8.2	10.1	7.8	15245
005	JWB	536	867	376	0.1	146	177	111	1.3	8.7	14.1	16.4	16090.7
006	HMZ	485	806	344	0.1	121	164	88	0.9	6.9	11.4	15.4	14558.6
007	BTI	560	830	402	0.1	112	150	89	0.5	6.7	10	14.3	16798.3
008	ASD	674	1108	436	0.1	133	190	95	0.8	7.8	12.7	22.5	20224.2
009	JFC	570	854	359	0.1	125	167	95	0.4	7.4	11.1	16.6	17113.6
010	BDK	562	897	373	0.1	139	177	107	1.2	8.3	13.2	17.5	16852.3
AVG		**550.20**	**828.20**	**386.70**	**0.10**	**130.40**	**163.40**	**102.30**	**1.80**	**7.77**	**11.65**	**14.77**	**16508.47**

SE		20.63	44.88	13.53	0.00	3.06	4.89	3.36	0.88	0.21	0.44	1.36	619.02
Study 2		**Wingate**											
		CHO/HAA											
Subject#	**Initials**	**Mean Watts**	**Peak Watts**	**Min. Watts**	**Peak Watts reached @ (sec)**	**Mean RPM**	**Peak RPM**	**Min. RPM**	**Peak RPM reached @ (sec)**	**Anaerobic Capacity (w/kg)**	**Anaerobic Power (w/kg)**	**Fatigue Index (w/s)**	**Total Work (joules)**
001	JB	501	716	371	0.1	123	144	102	1	7.5	10.7	11.5	15019.5
002	DGL	617	980	447	0.1	136	173	104	1	8.1	12.9	17.8	18515.9
003	JSW	451	673	316	0.1	130	158	101	1	7.5	11.2	12	13520.7
004	JLM	511	667	407	0.1	138	153	118	2.5	8.3	10.8	8.7	15325.6
005	JWB	537	878	381	0.1	145	172	116	1.4	8.7	14.2	16.6	16111
006	HMZ	487	891	336	0.1	122	166	87	0.7	6.9	12.6	18.5	14618.5
007	BTI	678	910	549	0.1	130	137	118	0.7	8.2	11	12	20330.6
008	ASD	656	1162	447	0.2	128	179	95	1.1	7.5	13.4	24	19676.5
009	JFC	579	880	439	0.1	124	155	99	0.7	7.5	11.4	14.7	17377.3
010	BDK	545	853	387	0.1	134	174	108	0.6	8	12.5	15.6	16350.8
AVG		**556.20**	**861.00**	**408.00**	**0.11**	**131.00**	**161.10**	**104.80**	**1.07**	**7.82**	**12.07**	**15.14**	**16684.64**
SE		**23.68**	**47.47**	**21.10**	**0.01**	**2.32**	**4.42**	**3.26**	**0.18**	**0.17**	**0.38**	**1.39**	**710.38**

Study 2		Serum Glucose Concentration (mmol/L)						
				CHO				
Subject #	**Initials**	**Pre**	**Ex-End**	**R30**	**R120**	**R150**	**R240**	**Wingate**
1	JB	5.51	4.04	10.08	6.53	7.43	5.16	3.96
2	DGL	4.29	2.58	6.99	4.70	6.20	4.48	3.25
3	JSW	4.78	5.55	8.45	3.84	5.65	2.87	4.20
4	JLM	4.08	4.26	5.72	4.50	7.39	4.36	2.71
5	JWB	4.40	4.80	5.85	3.00	5.82	5.27	4.33
6	HMZ	4.22	5.65	7.37	6.46	6.96	5.87	4.62
7	BTI	4.39	4.36	7.29	3.65	6.19	3.21	2.91
8	ASD	4.91	2.92	6.17	6.31	6.24	4.27	2.54
9	JFC	4.52	5.06	6.77	6.48	5.65	4.64	3.86
10	BDK	4.74	4.37	8.69	3.54	5.54	4.01	2.81
AVG		**4.58**	**4.36**	**7.34**	**4.90**	**6.31**	**4.42**	**3.52**
SE		**0.13**	**0.32**	**0.44**	**0.45**	**0.23**	**0.29**	**0.24**
				CHO/LAA				
Subject #	**Initials**	**Pre**	**Ex-End**	**R30**	**R120**	**R150**	**R240**	**Wingate**
1	JB	4.73	2.57	7.53	4.53	7.03	3.46	3.03
2	DGL	4.43	2.79	6.39	5.49	6.01	4.51	3.04
3	JSW	6.12	5.80	6.64	3.54	5.94	2.86	3.93
4	JLM	4.22	4.86	5.46	5.18	3.80	3.40	3.56
5	JWB	4.45	4.89	7.76	3.62	4.41	2.23	3.11
6	HMZ	4.78	5.50	7.39	5.35	5.38	4.54	3.83
7	BTI	5.48	4.16	7.76	4.53	5.13	3.07	2.99
8	ASD	4.46	4.22	5.71	3.22	4.20	3.06	2.19
9	JFC	4.55	4.77	6.69	6.05	6.37	4.45	4.52
10	BDK	5.00	3.64	7.87	5.40	6.12	4.81	2.89
AVG		**4.82**	**4.32**	**6.92**	**4.69**	**5.44**	**3.64**	**3.31**
SE		**0.18**	**0.34**	**0.28**	**0.30**	**0.33**	**0.28**	**0.21**
				CHO/HAA				
Subject #	**Initials**	**Pre**	**Ex-End**	**R30**	**R120**	**R150**	**R240**	**Wingate**
1	JB	4.59	3.45	8.30	4.78	6.11	4.09	2.19
2	DGL	4.57	2.35	6.21	5.69	6.24	4.19	4.75
3	JSW	5.02	5.10	7.65	4.13	6.41	3.38	3.92
4	JLM	4.76	4.18	5.16	4.87	6.62	4.52	3.41
5	JWB	4.96	4.50	7.33	3.01	5.76	3.32	2.80
6	HMZ	4.64	5.74	7.51	4.96	5.08	3.97	4.34
7	BTI	4.51	4.43	7.87	2.86	5.08	2.33	3.19
8	ASD	4.73	4.52	6.82	4.76	4.01	3.31	1.97
9	JFC	4.52	3.88	5.86	6.66	5.21	4.46	4.35
10	BDK	4.86	3.88	6.42	4.28	5.60	4.03	3.67
AVG		**4.72**	**4.20**	**6.91**	**4.60**	**5.61**	**3.76**	**3.46**
SE		**0.06**	**0.29**	**0.31**	**0.36**	**0.25**	**0.21**	**0.30**

Study 2		Serum Insulin Concentration (pmol/L)						
				CHO				
Subject #	**Initials**	**Pre**	**Ex-End**	**R30**	**R120**	**R150**	**R240**	**Wingate**
1	JB	149.94	45.63	430.38	274.88	746.03	357.95	151.12
2	DGL	89.45	43.13	157.30	63.55	260.51	174.39	102.92
3	JSW	104.66	62.99	277.17	88.48	217.73	64.93	48.41
4	JLM	80.56	52.99	196.40	70.21	351.49	84.94	54.73
5	JWB	122.30	94.87	346.56	108.20	514.49	256.83	122.30
6	HMZ	125.64	83.13	458.37	248.63	613.38	479.48	184.18
7	BTI	188.00	80.98	352.67	162.72	531.01	177.79	102.86
8	ASD	71.46	40.84	110.36	156.89	326.14	148.14	103.69
9	JFC	86.74	58.87	342.51	246.48	228.35	128.71	105.79
10	BDK	69.51	62.57	182.70	85.25	218.35	150.94	113.86
AVG		**108.83**	**62.60**	**285.44**	**150.53**	**400.75**	**202.41**	**108.99**
SE		**12.04**	**5.82**	**37.72**	**25.47**	**59.49**	**40.69**	**12.63**
				CHO/LAA				
Subject #	**Initials**	**Pre**	**Ex-End**	**R30**	**R120**	**R150**	**R240**	**Wingate**
1	JB	103.69	30.07	226.48	114.25	390.38	165.50	125.29
2	DGL	120.98	36.81	205.71	198.07	342.53	110.36	71.46
3	JSW	371.00	60.28	263.63	73.20	208.14	52.78	50.56
4	JLM	104.31	64.59	250.99	188.42	191.13	111.68	72.16
5	JWB	106.12	68.13	476.98	127.09	348.01	118.62	81.88
6	HMZ	144.39	105.22	737.00	271.34	773.74	223.56	135.36
7	BTI	97.65	89.87	553.86	223.77	834.65	207.72	104.87
8	ASD	88.90	52.78	232.17	95.84	295.58	128.48	59.31
9	JFC	96.05	82.40	249.44	262.94	469.06	271.06	150.01
10	BDK	100.86	65.56	179.53	88.32	306.65	146.58	94.52
AVG		**133.39**	**65.57**	**337.58**	**164.32**	**415.99**	**153.63**	**94.54**
SE		**26.86**	**7.25**	**58.91**	**23.36**	**69.68**	**20.40**	**10.63**
				CHO/HAA				
Subject #	**Initials**	**Pre**	**Ex-End**	**R30**	**R120**	**R150**	**R240**	**Wingate**
1	JB	115.70	40.49	471.50	402.32	921.05	298.22	95.77
2	DGL	101.81	39.38	201.89	206.75	303.57	183.97	211.41
3	JSW	97.37	40.70	382.67	83.62	165.85	81.26	68.82
4	JLM	111.81	40.70	193.28	160.43	452.12	150.36	82.65
5	JWB	114.52	67.23	622.97	122.93	876.32	146.75	102.92
6	HMZ	121.75	105.63	870.63	367.53	1375.60	213.98	193.14
7	BTI	174.11	145.22	858.75	286.48	1526.51	175.29	142.30
8	ASD	146.33	47.71	364.96	169.25	329.68	204.81	79.94
9	JFC	67.05	70.63	201.50	303.67	486.91	262.28	252.70
10	BDK	66.67	66.39	160.06	62.03	152.44	87.12	136.61
AVG		**111.71**	**66.41**	**432.82**	**216.50**	**659.00**	**180.40**	**136.63**
SE		**10.30**	**10.98**	**85.41**	**37.35**	**155.96**	**21.84**	**19.95**

Study 2		Plasma Lactate Concentration (mmol/L)						
CHO								
Subject #	**Initials**	**Pre**	**Ex-End**	**R30**	**R120**	**R150**	**R240**	**Wingate**
1	JB	1.14	7.56	1.84	1.37	1.40	1.37	7.39
2	DGL	0.97	5.37	1.53	0.94	0.91	1.28	4.92
3	JSW	1.11	12.01	2.07	1.23	1.16	1.10	8.61
4	JLM	1.46	6.53	1.35	1.06	0.97	0.78	4.47
5	JWB	0.76	10.09	1.88	0.93	1.00	0.87	5.30
6	HMZ	0.61	8.02	2.38	0.82	0.87	1.05	2.77
7	BTI	1.61	4.29	2.31	1.57	1.29	0.96	5.09
8	ASD	0.66	2.81	1.77	0.94	0.92	0.97	3.53
9	JFC	0.89	11.41	2.67	1.27	1.05	0.84	2.43
10	BDK	1.15	4.41	1.56	1.21	1.39	1.47	7.09
AVG		**1.04**	**7.25**	**1.93**	**1.14**	**1.10**	**1.07**	**5.16**
SE		**0.10**	**1.00**	**0.13**	**0.07**	**0.06**	**0.07**	**0.64**
CHO/LAA								
Subject #	**Initials**	**Pre**	**Ex-End**	**R30**	**R120**	**R150**	**R240**	**Wingate**
1	JB	0.93	4.67	1.77	1.12	1.15	1.13	6.13
2	DGL	0.96	5.62	1.60	1.26	1.01	1.09	5.15
3	JSW	1.31	10.54	2.57	1.18	1.34	1.12	5.49
4	JLM	1.42	10.35	2.70	1.31	1.06	1.10	7.00
5	JWB	1.62	11.68	3.27	2.58	1.84	1.87	6.94
6	HMZ	0.72	9.10	1.68	1.09	1.03	1.09	3.90
7	BTI	1.46	4.75	1.98	1.64	1.46	1.56	3.40
8	ASD	0.75	8.54	1.78	1.42	0.84	0.89	11.16
9	JFC	0.56	7.24	1.90	1.32	0.99	0.88	2.58
10	BDK	0.93	4.94	1.79	1.70	1.44	1.32	7.88
AVG		**1.07**	**7.74**	**2.10**	**1.46**	**1.22**	**1.21**	**5.96**
SE		**0.11**	**0.84**	**0.17**	**0.14**	**0.09**	**0.10**	**0.79**
CHO/HAA								
Subject #	**Initials**	**Pre**	**Ex-End**	**R30**	**R120**	**R150**	**R240**	**Wingate**
1	JB	0.99	8.19	1.99	1.67	1.54	1.26	5.25
2	DGL	1.23	5.08	1.51	0.95	1.02	1.07	4.22
3	JSW	0.97	11.35	2.24	1.17	1.41	0.92	7.74
4	JLM	1.84	6.49	1.53	1.28	1.23	1.23	7.90
5	JWB	0.69	8.30	1.52	1.08	1.08	0.94	4.73
6	HMZ	0.67	10.14	2.10	1.22	0.94	0.91	10.09
7	BTI	1.48	7.83	2.04	1.28	1.32	1.15	5.84
8	ASD	1.06	9.32	1.85	1.20	1.09	1.10	6.89
9	JFC	0.63	7.22	2.26	1.13	0.83	0.80	2.28
10	BDK	0.85	5.69	2.31	1.20	1.19	1.27	8.67
AVG		**1.04**	**7.96**	**1.93**	**1.22**	**1.16**	**1.06**	**6.36**
SE		**0.12**	**0.62**	**0.10**	**0.06**	**0.07**	**0.05**	**0.74**

Study 2		Serum Creatine Kinase Concentration (nkat/L)					
		CHO		CHO/LAA		CHO/HAA	
Subject #	Initials	Pre	24hrs	Pre	24hrs	Pre	24hrs
1	JB	2307.66	4648.29	4521.92	6585.99	2448.69	7893.67
2	DGL	1333.32	1734.41	1192.29	3102.52	1633.68	2139.17
3	JSW	1217.93	2036.60	1483.50	1934.04	1749.06	2373.59
4	JLM	1300.35	2109.86	1725.25	2595.20	1611.70	1899.24
5	JWB	1291.19	1890.08	1410.24	1617.19	1152.00	1597.05
6	HMZ	1983.49	3913.87	652.01	1164.82	749.07	1137.35
7	BTI	1864.44	3531.09	1672.14	3494.46	1655.66	3457.83
9	JFC	2239.90	3536.58	1655.66	1767.38	1569.58	4948.65
10	BDK	1741.73	1987.15	1384.60	3406.55	1362.62	2049.42
AVG		**1697.78**	**2820.88**	**1744.18**	**2852.02**	**1548.01**	**3055.11**
SE		**142.57**	**361.58**	**363.92**	**542.40**	**153.87**	**713.11**

Study 2		Serum Myoglobin Concentration (ng/ml)			
		CHO			
Subject #	**Initials**	**Pre**	**Ex-End**	**R120**	**Win**
1	JB	18.29	49.45	77.81	75.53
2	DGL	33.97	37.39	63.12	64.32
3	JSW	46.66	78.42	65.53	62.72
4	JLM	47.76	57.99	82.30	89.80
5	JWB	41.92	36.28	70.27	61.97
6	HMZ	33.34	35.62	76.17	53.52
7	BTI	13.17	23.51	41.61	33.70
8	ASD	14.53	48.34	45.28	61.61
9	JFC	20.35	23.48	47.10	60.63
10	BDK	25.43	25.60	36.33	37.88
AVG		**29.54**	**41.61**	**60.55**	**60.17**
SE		**4.13**	**5.50**	**5.27**	**5.15**
		CHO/LAA			
Subject #	**Initials**	**Pre**	**Ex-End**	**R120**	**Win**
1	JB	36.23	62.80	105.50	86.79
2	DGL	33.97	40.89	85.37	68.79
3	JSW	39.84	62.32	83.61	53.76
4	JLM	56.28	40.78	43.37	56.74
5	JWB	29.79	27.55	39.58	32.04
6	HMZ	22.51	20.65	31.72	30.75
7	BTI	7.59	26.18	156.10	106.51
8	ASD	40.91	37.60	41.92	35.30
9	JFC	12.99	13.74	19.57	19.57
10	BDK	24.31	32.94	83.64	58.99
AVG		**30.44**	**36.55**	**69.04**	**54.92**
SE		**4.52**	**5.13**	**13.21**	**8.58**
		CHO/HAA			
Subject #	**Initials**	**Pre**	**Ex-End**	**R120**	**Win**
1	JB	25.86	62.18	254.52	220.79
2	DGL	30.95	30.62	38.09	49.24
3	JSW	19.36	33.10	61.39	50.24
4	JLM	35.33	42.32	49.24	51.11
5	JWB	25.65	24.39	43.93	41.58
6	HMZ	17.57	23.14	20.96	39.91
7	BTI	22.06	43.24	53.29	38.72
8	ASD	24.39	35.95	36.94	33.34
9	JFC	12.24	30.98	213.55	168.21
10	BDK	20.47	20.47	45.93	29.08
AVG		**23.39**	**34.64**	**81.78**	**72.22**
SE		**2.10**	**3.89**	**25.78**	**20.88**

Study 2		Muscle Glycogen Concentration (µmol/g wet muscle)								
		CHO			CHO/LAA			CHO/HAA		
Subject #	Initials	Pre	Post	Difference	Pre	Post	Difference	Pre	Post	Difference
1	JB	26.41	38.44	12.03	17.33	32.80	15.46	13.68	26.22	12.55
2	DGL	10.87	38.50	27.63	6.13	21.51	15.39	10.35	33.01	22.66
3	JSW	50.79	75.84	25.05	48.84	65.71	16.87	35.68	52.55	16.86
4	JLM	49.12	68.44	19.32	56.29	68.83	12.53	73.86	80.90	7.04
5	JWB	12.54	32.48	19.94	36.13	52.93	16.80	21.71	39.63	17.92
6	HMZ	14.04	37.57	23.53	33.82	60.24	26.42	29.70	45.57	15.87
7	BTI	17.87	41.46	23.59	30.81	54.39	23.58	26.59	53.38	26.79
8	ASD	18.49	42.08	23.59	27.06	37.50	10.45	32.38	40.87	8.49
9	JFC	28.32	50.68	22.36	48.71	62.85	14.14	8.97	25.16	16.20
10	BDK	34.78	52.78	18.00	30.07	59.70	29.64	32.12	41.86	9.74
AVG		**26.32**	**47.83**	**21.50**	**33.52**	**51.65**	**18.13**	**28.50**	**43.92**	**15.41**
SE		**4.60**	**4.51**	**1.39**	**4.79**	**4.98**	**1.99**	**5.89**	**5.11**	**1.97**

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